METHOD DEVELOPMENT AND VALIDATION OF SITAGLIPTIN AND SIMVASTATIN IN TABLET DOSAGE FORM BY RP-HPLC

DISSERTATION

Submitted to

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In partial fulfilment for the award of the degree of

MASTER OF PHARMACY
In
(Pharmaceutical Analysis)

By

26101132

Under the Guidance of

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DECLARATION

The thesis entitled **"METHOD DEVELOPMENT AND VALIDATION OF SIMVASTATIN AND SITAGLIPTIN BY RP-HPLC METHOD"** was carried out by me in Department of Pharmaceutical Analysis, C.L.Baid Metha College of Pharmacy, Chennai – 97 during the academic year 2011-2012. The work embodied in this thesis is original, and is not submitted in part or full for any other degree of this or any other University.

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Date: DEPT OF PHARMACEUTICALANALYSIS

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It's my privilege to express my grateful and sincere gratitude to **Dr. Grace Rathnam, M.Pharm. , Ph.D** principal, HOD, department of pharmaceutics. C.L.Baid Metha College of Pharmacy.

I acknowledge my sincere thanks **to Mrs. G.UMA, M.Pharm**. assistant professor, **Mrs. R.VIJAYA GEETHA, M.Pharm(PhD),** assistant professor, **Mrs. .S. YAMUNA M.Pharm (PhD),** assistant professor Department of pharmaceutical Analysis, for their valuable suggestions throughout my thesis work.

I thank almighty and my parents for blessing with happiness and I convey my thanks to all my friends who helped me in all the issues they can.

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LIST OF ABBREVIATIONS USED

ICH	-	International Conference on Harmonization
I.P	-	Indian pharmacopeia.
λ	-	Lambda
LOD	-	Limit of Detection
LOQ	-	Limit of Quantification
µg/ml	-	Microgram per Milliliter
mg/tab	-	Milligram Per tablet
mL	-	Millilitre
nm	-	Nanometer
%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
S.D.	-	Standard Deviation
S.E.	-	Standard Error
USP	-	United States Pharmacopoeia
IR	-	Infra Red
UV-VIS	-	Ultraviolet- Visible
°C	-	Degree Celsius
gms	-	Grams
µl	-	Microlitre
rpm	-	Revolutions Per Minute
µ	-	Micron
v/v	-	Volume/Volume
USP	-	United State Pharmacopeia
BP	-	British Pharmacopeia
ng	-	nano gram
STG	-	sitagliptin
SMV	-	simvastatin

Chapter-1

introduction

INTRODUCTION [1]

1.1. Analytical chemistry

Pharmaceutical Analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. The term "Pharmaceutical analysis" is otherwise called quantitative pharmaceutical chemistry. Pharmaceutical analysis includes both qualitative and quantitative analysis of drugs and pharmaceutical substances starts from bulk drugs to the finished dosage forms. In the modern practice of medicine, the analytical methods are used in the analysis of chemical constituents found in human body whose altered concentrations during disease states serve as diagnostic aids and also used to analyze the medical agents and their metabolites found in biological system.

The term "quality" as applied to a drug product has been defined as the sum of all factors, which contribute directly or indirectly to the safety, effectiveness and reliability of the product. These properties are built into drug products through research and during process by procedures collectively referred to as "**quality control**".

Quality control guarantees within reasonable limits that a drug products

Is free of impurities.
Is physically and chemically stable
Contains the amount of active ingredients as stated on the label and
Provides optimal release of active ingredients when the product is administered.

Types of analytical methods

Analytical methods can be separated into classical and instrumental. classical methods (also known as wet chemistry methods) use separations such as precipitation, extraction, and distillation and qualitative analysis by color, odor, or melting point. Quantitative analysis is achieved by measurement of weighed or volume.

Instrumental methods use an apparatus to measure physical quantities of the analyte such as light_absorption, fluorescence, or conductivity. The separation of materials is accomplished using chromatography or electrophoresis methods.

Classification

Classical methods

Titration Reaction types

1.Acid-Base Titrations

Types of acid-base titrations.

i Strong Acid / Strong Base: pH at equivalence point = 7.

ii Weak Acid / Strong Base: pH at equivalence point >7.

iii Strong Acid / Weak Base: pH at equivalence point <7.

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i. Weighed drug after extraction.

ii. Weighed a derivative after separation.

iii. Weighed residue after ignition.

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iii. Coulometry.

iv.Electrogravimetry.

v. Conductance techniques.

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ii. Paper chromatography.

iii. Column chromatography.

iv.Gas chromatography.

v. High Performance Liquid Chromatography and high performance thin layer chromatography.

4. Miscellaneous methods.

i. Thermal analysis.

ii. Kinetic techniques.

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5. Hyphenated techniques.

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ii. LC-MS (Liquid chromatography – Mass spectrometry).

iii. GC-IR (Gas chromatography – Infrared spectroscopy).

iv. ICP-MS (Inductively coupled plasma – Mass spectrometry).

CHROMATOGRAPHY INTRODUCTION[2]

Chromatography is the name applied to a group of analytical techniques for isolation of components in mixture for qualitative identification, purification and quantitative estimation of many components.

Recently IUPAC has defined the chromatography as "A method used primarily for separation of the components of a mixture sample in which the components are separated and distributed between stationary phase and mobile phase. The stationary phase may be solid or liquid supported on a solid or gel and maybe packed in a column separated as a layer or distributed as a film, the mobile phase maybe gaseous or liquid."

The importance of Chromatography is increasing rapidly in pharmaceutical analysis. The exact differentiation, selective identification and quantitative determination of structurally closely related compounds are possible with chromatography. Another important field of application of chromatographic methods is the purity testing of final products and intermediates (detection of decomposition products and by products). As a consequence of the above points, chromatographic methods are occupying an ever-expanding position in the latest editions of the pharmacopoeias and other testing standards.

The modern form of column chromatography has been called high performance, high pressure, high resolution and high speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column

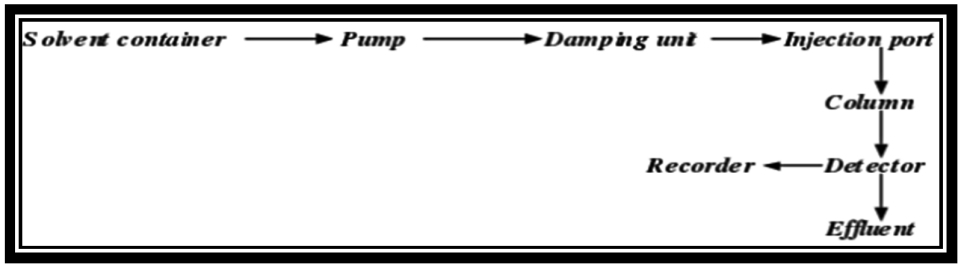
chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially.

High performance liquid chromatography is the fastest growing analytical technique for the analysis of drugs. Its simplicity, high specificity and wide range of sensitivity made it ideal for the analysis of many drugs in both dosage forms and biological fluids. This technique is based on the same methods of separation as that of classical column chromatography i.e. adsorption, partition, ion exchange and gel permeation, but it differs from the column chromatography in the fact that the mobile phase is passed through the packed column under high pressure. In classical open column chromatography, the mobile phase flows slowly through the column by means of gravity with the diameter of particles (of solid support) in the range of 150 - 200µm. But in HPLC, the separation is about 100 times faster than the conventional liquid chromatography due to packing of particles in the range of 3-10µm. Thus HPLC is having advantages of improved resolution, faster separation, improved accuracy, precision and sensitivity.

1.2.HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) [3]

The term 'Chromatography' covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

Instrumentation of HPLC



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Principle of separation in HPLC

The principle of separation in normal phase mode and reverse phase mode is adsorption. When a mixture of components is introduced in to a column, they travel according to their relative affinities towards stationary phase. The component which has more affinity towards the adsorbent travels slower. The component which has less affinity towards stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated HPLC is an analytical technique for resolution of solutes, in which separation is made by differential migration in a porous medium and migration is caused by flow of solvent.

Chromatography is one of the widely used physiochemical methods of separation of inorganic and organic substances related in their composition and properties. Chromatography is characterized by two important features,

a. A very large interface area, and

b. A dynamic way of operation, which ensures a more effective separation of substances as compared to other methods.

Modes of Chromatography

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography).

Advantages of HPLC

- It is efficient, highly selective and widely applicable.
- Only small sample amount is required for analysis.
- Ordinarily nondestructive to sample.
- HPLC readily adaptable to Quantitative analysis.
- It is simple and inexpensive equipment compared to GC.
- HPLC can accommodate nonvolatile and thermally unstable samples.
- It is generally applicable to inorganic ions.

Types of HPLC

Based on the mode of separation

Normal phase chromatography

Reverse phase chromatography.

Partition chromatography.

Displacement chromatography.

Based on principle of separation

Adsorption chromatography.

Ion exchange chromatography.

Size exclusion chromatography.

Affinity chromatography.

Chiral phase chromatography.

Ion pair chromatography.

Based on elution technique

Isocratic separation.

Gradient separation.

Based on the scale of operation

Analytical HPLC.

Preparative HPLC.

Based on the type of analysis

Qualitative analysis.

Quantitative analysis.

Based on the mode of separation

Normal Phase Chromatography

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents.

The silica structure is saturated with silanol groups at the end. These OH groups are statistically disturbed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase. This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present.

Dipole-induced dipole

Dipole-dipole

Hydrogen bonding

π-Complex bonding

These situations arise when the molecule has one or several atoms with lone pair electron or a double bond. The absorption strengths and hence k' values (elution series) increase in the following order- Saturated hydrocarbons < olefins < aromatics < organic halogen compounds < sulphides < ethers< esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional groups in

the sample molecule but also on steric factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.

Chemically modified silica, such as the aminopropyl, cyanopropyl and diol phases are useful alternatives to silica gel as stationary phase in normal phase chromatography.

The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phases and thus offer additional options for the optimizations of separations. Other advantages of bonded phases lie in their increased homogeneity of the phase surface.

Resolution with water in weak mobile phase may be most conveniently achieved by drying the solvents and then adding a constant concentration of water or some very polar modifier such as acetic acid or triethylamine (TEA) to the mobile phase. The addition of such polar modifiers serves to deactivate the more polar shape as well as the reproducibility of the retention times.

Reversed Phase Chromatography

Since 1960's chromatographers started modifying the polar nature of silanol group is chemically reacting silica with organic silanes. The objective is to made less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography.

A large number of chemically bonded stationary phases based on silica are available commercially. Silica based stationary phases are still most popular in reversed phase chromatography however other absorbents based on polymer (styrene-di-vinyl benzene copolymer) are slowly gaining ground.

The retention time decreases in the following order: aliphatics > induced dipoles (i.e. CCl_4) > permanent dipoles (e.g.CHC_{l3}) > weak Lewis bases (ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases.

As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimentional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

Partition chromatography

Partition chromatography was the first kind of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid-liquid applications. Partition chromatography uses a retained solvent, on the surface or within the grains or fibers of an “inert” solid supporting matrix as with paper chromatography, or takes advantage of some additional columbic and/or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a liquid stationary phase and the eluent separate

analytes based on the polar differences is known as Hydrophilic Interaction Chromatography (HILIC). Partition HPLC has been used historically on unbounded silica or alumina supports. Each works effectively for separating analytes by relative polar differences. However, HILIC has the advantage of separating acidic, basic and neutral solutes in a single chromatogram.

Eg CPC (Centrifugal Partition Chromatography), GLC (Gas Liquid Chromatography), and paper partition chromatography.

Displacement Chromatography

The basic principle of displacement chromatography is a molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites and thus displace all molecules with lesser affinities. In elution mode, substances typically emerge from a column in narrow Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds and thereby be resolved there must be substantial differences in some interaction between the bio molecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with the gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than “peaks”.

Based on principle of separation

Adsorption chromatography

When a mixture of compounds (adsorbate) dissolved in the mobile phase (eluent) moves through a column of stationary phase (adsorbent) they travel according to their relative affinities. The compound which has more affinity towards stationary phase travels slower and if less affinity towards stationary phase travels faster.

Eg GSC (gas-solid chromatography), TLC (thin layer chromatography), C.C (column chromatography), HPLC (High performance liquid chromatography).

Ion exchange chromatography

Ion-exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. It is the process by which a mixture of similar charged ions can be separated using ion exchange resin. There is a reversible exchange of ions between the ions present in the column. And those present in the ion exchange resin. For cations, cation exchange resin, for anions, an anion exchange resin is used.

Eg Cationic exchangers

Natural Inorganic - Zeolite, Clays.

Natural Organic - Peat, Lignite, Neutral Sulphonated coal.

Synthetic Inorganic - MgO, SiO_2.

Synthetic Organic - Polymeric Resin matrix containing acidic exchange resins.

Eg Anionic exchangers

Natural Inorganic - Dolamite.

Synthetic Inorganic - Heavy metal silicates.

Synthetic Organic - Polymeric Resin matrix containing basic exchange resins.

Size Exclusion chromatography

Size-exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, the large molecules enable to enter the pores eluting first. It is the process by which mixture of compounds with molecular sizes are separated by using gels. The gel used acts as molecular sieve. It can be separated by steric and diffusion effects of pores in the gels. The compound can separate according to the molecular sizes and the stationary phase is porous matrix.

Eg Separation of proteins, and polysaccharides.

Chiral phase chromatography

In this type of chromatography separation of optical isomers can be done by using chiral stationary phase i.e. levo and dextro form can be separated by using chiral stationary phases.

Eg chemically bonded silica gel.

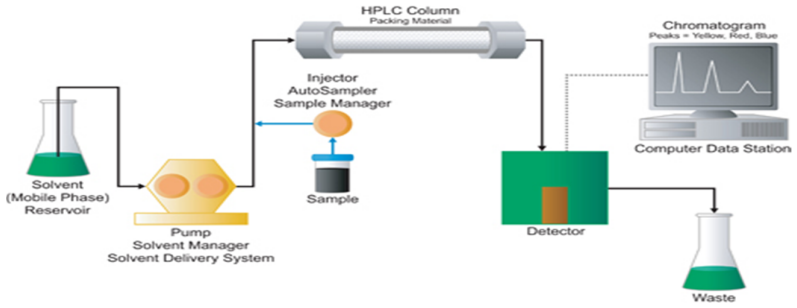
Ion pair chromatography

In this chromatography, a reverse phase column is converted temporarily into ion exchange column by using ion pairing agents like pentane or hexane.

Based on elution technique

Isocratic elution

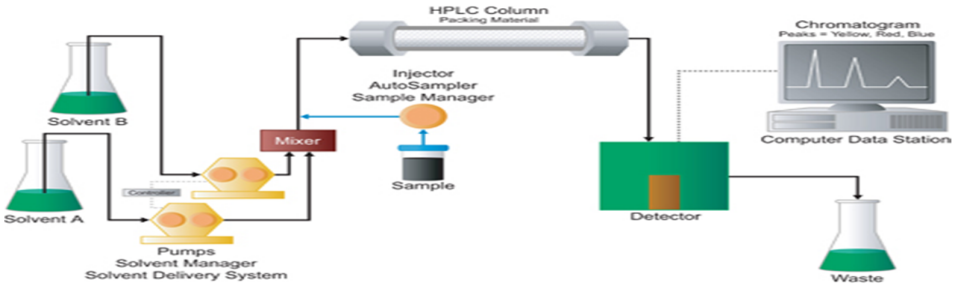
In this elution technique, a single mobile phase is pumped to the column throughout the analysis. The composition or the concentration of the mobile phase is not altered throughout the run.



Isocratic mode

Gradient elution

In gradient elution, the mobile phase is changed at regular time intervals depending upon the sample of analysis. The concentration of the stronger mobile phase is increased during the run. Preparative chromatography is usually carried out by gradient elution technique.



Gradient mode

Based on the scale of operation[4]

Analytical HPLC

Where it is used analysis of samples are done. But recovery of samples for reusing is normally not done, since the sample used is very low.

Eg microgram quantities.

Preparative HPLC

Where the individual fractions of pure compounds can be collected using fraction collector. And the collector samples are reused.

Eg separation of few grams of mixtures.

Based on the type of analysis

Qualitative Analysis

Which is used to identify the compound, detect the presence of impurities, to find out the number of components, etc. this is done using retention time values?

Quantitative Analysis

This is done to determine the quantity of the individual or several components in a mixture. This is done by comparing the peak area of the standard and sample. Chromatographic methods can be classified most practically according to the stationary and mobile phases, as shown in the table.

Stationary phase	Mobile phase	Method
Solid	Liquid	Adsorption column, thin-layer, ion exchange, High performance liquid chromatography.
Liquid	Liquid Gas	Partition, column, thin-layer, HPLC, paper chromatography. Gas – Liquid Chromatography.

Table-1 **Classification of Chromatographic methods**

The importance of Chromatography is increasing rapidly in pharmaceutical analysis. The exact differentiation, selective identification and quantitative determination of structurally closely related compounds are possible with chromatography. Another important field of application of chromatographic methods is the purity testing of final products and intermediates (detection of decomposition products and by-products). As a consequence of the above points, chromatographic methods are occupying an ever-expanding position in the latest editions of the pharmacopoeias and other testing standards.

The modern form of column chromatography has been called high performance, high pressure, high-resolution and high-speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially.

The essential equipment consists of an eluent, reservoir, a high-pressure pump, and an injector for introducing the sample, a column containing the stationary phase, a detector and recorder. The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multi component mixtures.

The systems used are often described as belonging to one of four mechanistic types, adsorption, partition, ion exchange and size-exclusion. Adsorption chromatography arises from interaction between solutes on the surface of the solid stationary phase. Partition chromatography involves a liquid stationary phase, which is immiscible with the eluent and coated on an inert support. Adsorption and partition systems can be normal phase (stationary phase more polar than eluent) or reversed phase (stationary phase less polar than eluent). Ion-exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. Size-exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, the large molecules enable to enter the pores eluting first.

Chromatography parameters [5]

Calculating the following values (which can be included in a custom report) used to access overall system performance.

1. Relative retention
2. Theoretical plates
3. Capacity factor
4. Resolution
5. Peak asymmetry
6. Plates per meter
7. Back pressure.

The parameters used to calculate these system performance values for the separation of two chromatographic components. (Note: Where the terms W and t both appear in the same equation they must be expressed in the same units).

Relative retention (Selectivity)

The relative retention is calculated as an estimate using the following formula:

$$\alpha = (t_2 - t_a) / (t_1 - t_a)$$

Theoretical plates (column efficiency)

It measures the band spreading of a peak. When band spread in smaller, the number of theoretical plates is higher. It indicates a good column and system performance.

$$n = 16 (t / W)^2$$

Capacity factor

This factor determines the retention of a solute and can be calculated from the chromatogram using the following formula

$$k' = (t_2 – t_a)/ t_a$$

A low k' value indicates that the peak elutes close to the solvent front, which may compromise selectivity. A minimum k' value of 1 is recommended for the peak of interest.

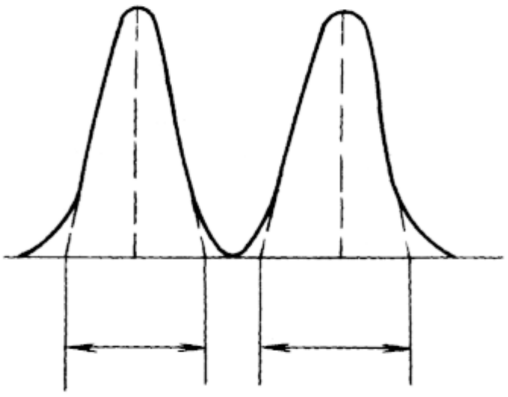
The retention time of the test substance can be varied, if necessary, by changing the relative proportion or composition of solvents in the mobile phase. Generally, an increase in the proportion of a more polar solvent will lead to a shorter retention time on a normal-phase column and a longer retention time on a reserved-phase column.

Resolution

It is measure of the extent of separation of two compounds and the baseline separation is achieved. The resolution between two peaks of similar height in a chromatogram can be calculated using the following formula:

$$R = 2 (t_2 - t_1) / (W_2 + W_1)$$

To ensure the accuracy of quantitative analysis, the *R* value of the analyte peak with the adjacent peak must be larger than 1.5, unless otherwise specified. (*t*R1 *t*R2
*W*1 *W*2)



Peak asymmetry (Tailing factor)

The Tailing Factor T, a measure of peak symmetry is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. In some cases, values less than 1 may be observed. As peak asymmetry increases integration and hence precision becomes less reliable.

$$\mathbf{T = W0.05 / 2f}$$

Where, W0.05 = peak width at 5% height

f = distance from peak front to apex point at 5% height.

The accuracy of Quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where / when the peak ends and hence the calculation of the area under the peak.

Plates per meter

Plates per meter can be expressed as

$$N = n / L$$

Backpressure

The pressure required to pump the mobile phase through the column. It is related to mobile phase viscosity (η), flow rate (F), column length (L), and diameter (dc), and particle size (dp) by the following equation:

$$\mathbf{\Delta P\ \alpha\ FL\eta\ /\ dp2\ dc2}$$

HETP (High Equivalent Theoretical Plates)

A theoretical plate can be of any height, which decides the efficiency of separation. If HETP is less the column is more efficient. If HETP is more, the column is less efficient. The height equivalent to a theoretical plate (HETP) is given by

$$HETP = L/n$$

Where, α = Relative retention.

t_2 = Retention time of the peak of interest measured from point of injection.

t_1 = Retention time of the reference peak measured from point of injection.

t_a = Retention time of an inert peak not retained by the column, measured from point of injection.

n = Theoretical plates.

t = Retention time of the component.

W = Width of the base of the component peak using tangent method.

K' = Capacity factor.

R = Resolution between a peak of interest (peak 2) and the peak preceding it

W_2 = Width of the base of component peak 2.

W_1 = Width of the base of component peak 1.

T = Peak asymmetry, or tailing factor.

W0.05 = Distance from the leading edge to the tailing edge of the peak, measured at a point 5 % of the peak height from the baseline.

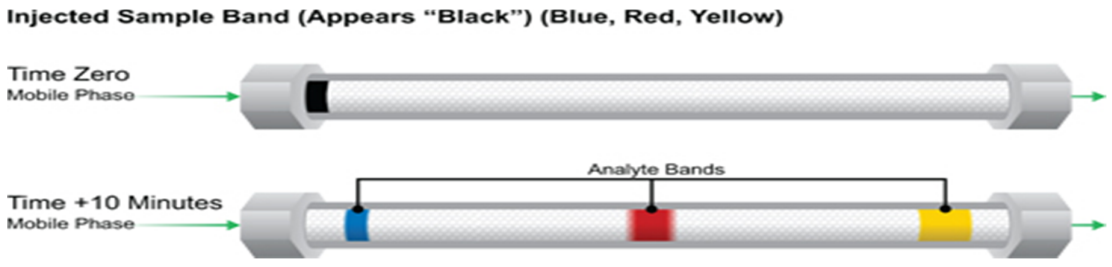
f = Distance from the peak maximum to the leading edge of the peak.

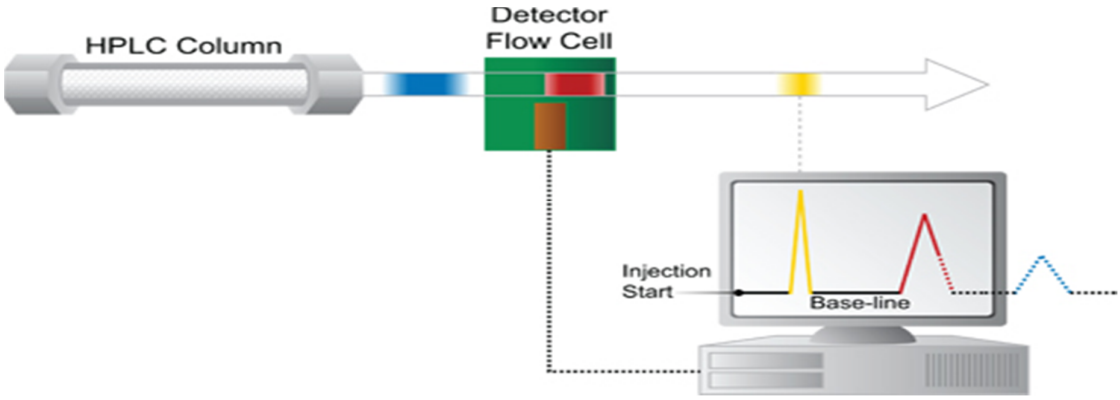
N = Plates per meter.

L = Column length, in meters.

The HPLC process starts with the preparation of sample and placing in a vial .the organic phase and buffer are selected and the temperature for the analysis should be set. the injection volume vial number should be set and the chromatogram development occurs based on the affinity towards the stationary phase.

HPLC PROCESS





HPLC METHOD VALIDATION [6]

1.3. ICH GUIDELINES

According to **USP** General Chapter <1225> "Validation is the process of providing documented evidence that the method does what it is intended to do". In other words the process of method validation ensures that the proposed analytical methodology is accurate, specific, reproducible and rugged for its intended use

According to the **FDA** Guidelines on General Principles of Process Validation, process validation is defined, '' As establishing documented evidence, which provides a high degree of assurance, that a specific process will consistently produce a product meeting its predetermined specifications and quality characteristics.''

According to **ICH Guidelines** Validation of an Analytical procedure is to demonstrate that it is suitable for its intended purpose.

Method validation can be defined as (ICHQ.2B) "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Method Validation, however, is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

TYPES OF ANALYTICAL PROCEDURES TO BE VALIDATED[7]

- Identification tests.
- Quantitative tests for impurities content
- Limit tests for the control of impurities
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Method Validation is required for the following

- Method validation required for assuring the quality identity purity.
- Achieving the acceptance of the product by the international agencies.
- Mandatory requirements for the registration of Pharmaceuticals pesticides and formations.
- Validation methods are only acceptable for under taking sufficiency testing.

Identification tests are indented to ensure the identity of analyte in a sample. This is normally achieved by comparison of sample chromatograms to that of a reference standard.

Testing for impurities can be either a qualitative or quantitative limit test for the impurity in the sample.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters.

1.4. STATISTICAL PARAMETERS FOR METHOD VALIDATION[8]

- **Recovery**
- **Response function.**
- **Sensitivity**
- **Precision.**
- **Accuracy.**
- **Calibration.**

- **Standard deviation of slope.**
- **Standard deviation of intercept.**
- **Correlation coefficient.**
- **Linearity and sensitivity of the method.**
- **Limit of detection.**
- **Limit of quantization.**
- **Ruggedness.**
- **Robustness.**
- **Stability**
- **Range .**

Recovery

The absolute recovery of analytical method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample. It is best established by comparing the responses of extracted samples at low, medium and high concentrations in replicates of at least 6 with those non-extracted standards, which represent 100 % recovery.

$$\text{Absolute recovery} = \frac{\text{Response of an analyte spike into matrix (processed)}}{\text{Response of analyte of pure standard (unprocessed)}} \times 100$$

If an internal standard is used, its recovery should be determined independently at the concentration levels used in the method.

Response function

In chromatographic methods of analysis, peak area or peak height may be used as response function to define the linear relationship with concentration known as the calibration model. It is essential to verify the calibration model selected to ensure that it adequately describes the relationship between response function (Y) and concentration (X).

Sensitivity

The method is said to be sensitive if small changes in concentration cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limits of quantification (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can determined with acceptable accuracy. It is suggested that, this be set at ± 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

Precision

The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels:

Repeatability/ System Precision.

Reproducibility/ Method Precision

Ruggedness/ Intermediate Precision.

The purpose of carrying out a determination is to obtain a valid estimate of a 'true' value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important criteria for judging analytical procedures by their results.

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The term 'set' is defined as referring to a number (n) of independent replicate measurements of some property. One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set. The standard deviation S, is given by

$$S = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Standard deviation has the same units as the property being measured.

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S × 100 / x

Acceptance: %RSD should be not more than 2% for assay.

Accuracy [9]

Accuracy of analytical method is 'Measure of how close the experimental value to the true value.'

Accuracy normally refers to the difference between the mean set of results and the true value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

Accuracy is best reported as percentage bias, which is calculated from the expression.

$$\%Bias = \frac{(measured\ value - true\ value)}{true\ value} X\ 100$$

Since for real samples the true value is not known, an approximation is obtained based on spiking drug – free matrix to a nominal concentration. The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug – free matrix sampler.

Accuracy studies is evaluated by recovery studies in which known amount of the drug substances is added to the previously analyzed pharmaceutical preparations of the drug and tested for the added drug.

Acceptance: The method is considered as accurate if average recovery is in between 97.0% to 103.0% for assay

Calibration

Calibration is the most important step in bioactive compound analysis. A good precision and accuracy can only be obtained when a good calibration procedure is adopted. In the spectrophotometric methods, the concentration of a sample cannot be measured directly, but is determined using another physical measuring quantity 'y' (absorbance of a solution). An unambiguous empirical or theoretical relationship can be shown between this quantity and the concentration of an analyte. The calibration between y = g (x) is directly useful and yields by inversion of the analytical calculation function.

The calibration function can be obtained by fitting an adequate mathematical model through the experimental data. The most convenient calibration function is linear, goes through the origin and is applicable over a wide dynamic range. In practice, however, many deviations from the ideal calibration line may occur. For the majority of analytical techniques the analyst uses the calibration equation.

$$Y = a + bx.$$

In calibration, univariate regression is applied, which means that all observations are dependent upon a single variable X.

Standard deviation of slope (Sb)

The standard deviation of slope is proportional to standard error of estimate and inversely proportional to the range and square root of the number of data points.

$$Sb = \sqrt{\frac{\sum_{i\ 1}^{p}(y_i \quad y_i)^2}{(n \quad 2)}} \quad \sqrt{\frac{1}{\sum_{i\ 1}^{p}(x_i \quad \bar{x}_i)^2}}$$

Where Xi is the arithmetic mean of Xi values.

Standard deviation of intercept (Sa)

Intercept values of least squares fits of data are often to evaluate addeditive errors between or among different methods.

Sa = $$\sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right)^2}{(n-2)}}\sqrt{\frac{1}{\sum_{i=1}^{n}(x_i - \bar{x}_i)^2}}\sqrt{\frac{\sum_{i=1}^{n} x_i^2}{n}}$$

Where Xi denote the arithmetic mean of xi, values.

Correlation Coefficient (r)

The correlation coefficient r(x, y) is more useful to express the relationship of the chosen scales. To obtain a correlation coefficient the covariance is divided by the product of the standard deviation of x and y.

r= $$\frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2 (y_i - \bar{y})^2}} \quad (n-1)$$

Linearity and sensitivity of the method

Knowledge of the sensitivity of the color is important and the following terms are commonly employed for expressing sensitivity. According to Bouger- Lambert – Beer's law, log intensity of incident radiations

$$A \quad Log\frac{Intensity\ of\ incident\ light}{Intensity\ of\ transmitted\ light}$$

The absorbance (A) is proportional to the concentration (c) of the absorbing species, if absorptivity (Î) and thickness of the medium (t) are constant. When c is in moles per liter, the constant is called molar absorptivity. Beer's law limits and Imax values are expressed as µg ml^{-1} and $mole^{-1}$ cm^{-1} respectively.

Sandell's sensitivity refers to the number of µg of the drug to be determining, converted to the colored product, which in a column solution of cross section 1cm2 shows an absorbance of 0.001 (expressed as µg cm^{-2}).

Limit of detection[10]

The Detection Limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily qualtitated as an exact value. The detection limit (LOD) may be expressed as

$$LOD = 3.3\sigma/S$$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte.

limit of quantization

LOQ is defined as the lowest concentration of the substance (analyte) in a sample that can be estimated quantitatively with acceptable precision, accuracy and reliability by a given method under stated experimental conditions. Quantitation Limit (LOQ) may be expressed as

$LOQ = 10\ \sigma\ /S$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

Ruggedness

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, source of reagents, chemicals, solvents etc. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Acceptance: %RSD should not be more than 2.0% for assay.

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as “a measure of its capacity to remain unaffected by small but deliberate variations in method parameters providing an indication of reliability during normal usage”. The robustness of a method is the ability to remain unaffected by small changes in parameters such as pH of the mobile phase, temperature, %organic solvent strength and buffer concentration etc. to determine the robustness of the method experimental conditions were

purposely altered and chromatographic characters were evaluated. System suitability parameters are to be met at different conditions.

Validation parameters for robustness

Parameter	Variation to be checked
Flow rate	± 0.2 mL/min
Column temperature	±5˚C
pH variation	±0.2
Mobile phase composition	±5%

Table-2

Stability

To generate reproducible and reliable results, the samples, standards and reagents used for the HPLC method must be stable for a reasonable time (e.g. one day, one week, one month etc, depending upon need). For example, the analysis of even a single sample may require ten or more chromatographic runs to determine the system suitability, including standard concentrations to create a working analytical curve and duplicate or triplicate injections of the sample to be assayed.

Range

The range of a method can be defined as the upper and lower concentrations for which the analytical method has adequate accuracy, precision and linearity. The range of concentrations examined will depend on the type of method and its use.

Chapter-2

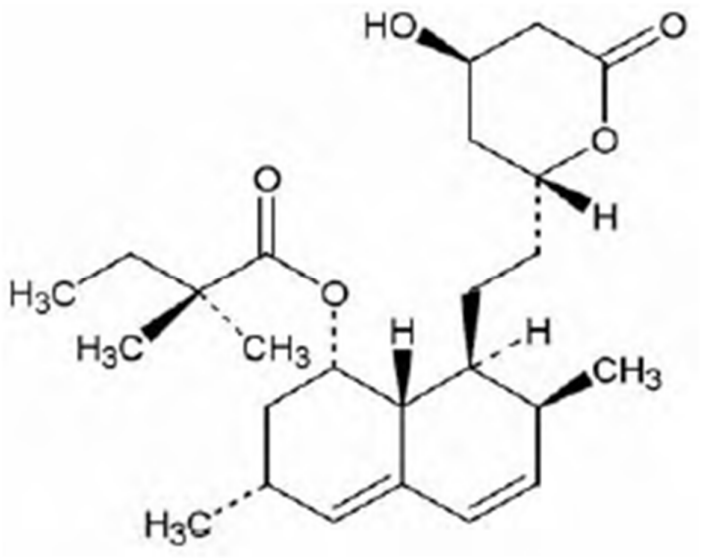
Drug profile

DRUG PROFILE

SIMVASTATIN[11]

A synthetic analog of lovastatin (antilipidemic).

CHEMICAL STRUCTURE



SIMVASTATIN

CHEMICAL FORMULA - $C_{25}H_{38}O_{5.}$

CHEMICAL NAME - (1S,3*R*,7*S*,8*S*,8a*R*)-8-{2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl}-3,7-dimethyl 1,2,3,7,8,8a-hexahydronaphthalen-1-yl2,2 dimethylbutane ate.

MOLECULAR WEIGHED - 418.56622

SOLUBILITY - practically insoluble in water, and freely soluble in chloroform, methanol and ethanol.

λMAX - 238nm.

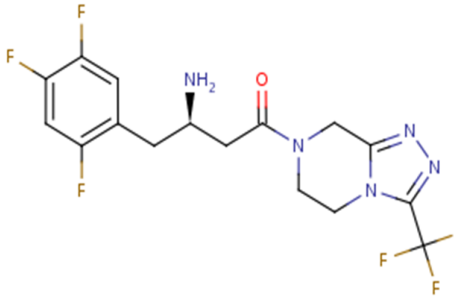
PKa - 4.3

MELTING POINT - 139°C.

STORAGE CONDITION	- It should be stored in well-closed, light- resistant containers at 5-30 deg C.
THERAPEUTIC ACTIVITY	- Used in the treatment of hypercholesterolemia. Hydroxy methyl CoA reductase, hypolipidemic agent.
DESCRIPTION	- white powder.
M.O.A	- The 6-membered lactone ring of Simvastatin is hydrolyzed in vivo to generate the Dihydroxy acid, an active metabolite structurally similar to HMG-CoA (hydroxy CoA). Once hydrolyzed, Simvastatin competes with HMG-CoA for HMG-CoA1 microsomal enzyme. Interference with the activity of this enzyme reduces the mevalonic acid, a precursor of cholesterol.

SITAGLIPTIN[12]

CHEMICAL STRUCTURE



SITAGLIPTIN

CHEMICAL FORMULA	-	***C*$_{16}$*H*$_{15}$*F*$_6$*N*$_5$*O*.**
CHEMICAL NAME	-	*(3R)-3-amino-1-[3-(trifluoromethyl)-5H,6H,7H,8H*-[1,2,4]triazolo[4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-one .
MOLECULAR WEIGHED	-	407.3136.
SOLUBILITY	-	It is soluble in water and N,N-dimethyl formamide; slightly soluble in methanol; very slightly soluble in ethanol, acetone, and acetonitrile; and insoluble in isopropanol and isopropyl acetate.
λ MAX	-	268nm.
PKa	-	7.7.
MELTING POINT	-	**252.*1***^{o}C
STORAGE CONDITION	-	stored in well closed container**.**
THERAPEUTIC ACTIVITY	-	***A***nti diabetic drug.
DESCRIPTION	-	white to off-white, crystalline, nonhygro scopic powder.
M.O.A[13]	-	Sitagliptin is a Dipeptidyl peptidase-4(DPP-4) inhibitor, which is believed to exert its actions in

patients with type2 diabetes by slowing the inactivation of incretin hormones. Concentrations of the active intact hormones are increased by SITAGLIPTIN, thereby increasing and prolonging the action of these hormones. Incretin hormones, including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are released by the intestine throughout the day, and levels are increased in response to a meal. These hormones are rapidly inactivated by the enzyme, DPP-4. The incretins are part of an endogenous system involved in the physiologic regulation of glucose homeostasis.

When blood glucose concentrations are normal or elevated, GLP-1 and GIP increase insulin synthesis and release from pancreatic beta cells by intracellular signaling pathways involving cyclic AMP. GLP-1 also lowers glucagon secretion from pancreatic alpha cells, leading to reduced hepatic glucose production. By increasing and prolonging active incretin levels, SITAGLIPTIN increases insulin release and decreases glucagon levels in the circulation in a glucose-dependent manner. Sitagliptin demonstrates selectivity for DPP-4 inhibitor.

Chapter-3

Literature review

LITERATURE REVIEW

1. **Sreelakshmy.N**[14] *et.al* (2011) developed a reverse phase high performance liquid chromatographic method for the simultaneous determination of Simvastatin and ezetimibe in tablet dosage form. The separation was effected on a symmetry C8 (4.68 x150mm,5 Âµm)using a mobile phase consisting of phosphate buffer and acetonitrile (50:50v/v)at a flow rate of 1ml/min. The detection was made at 236 nm. The retention time for ezetimibe and Simvastatin were 4.4 and 8.4 respectively. Caliberation curve were linear over the ranges of 10-50Âµg/ml for both ezetimibe and Simvastatin. The mean recovery was found to be 102.7% and 99.6% for ezetimibe and Simvastatin respectively. The correlation coefficients for both the components were close to. The developed method was validated according to ICH guidelines.
2. **Pravish Kumar Tiwari** [15] ***et.al*** **(2010)** has developed a simple, sensitive and validated HPTLC method to determine Niacin and Simvastatin simultaneously in synthetic mixture form. Chromatographic separation was achieved on a RP18 plate using a mixture of Methanol: Water: Acetic acid (60:40:0.1) at a wavelength of 237 nm. Linearity of the method was found to be in the concentration range of 5000.0-25000.0 µg/ml for niacin and 100.0-500.0 µg/ml for Simvastatin with correlation coefficient greater than 0.999.
3. **Jat R K**[16] ***et.al*** **(2012)** developed a simple, accurate rapid and precise RP-HPLC method and validated for determination of Simvastatin in bulk drug. The RP-HPLC separation was achieved on Promosil C-18, (250 mm, 4.6 mm, 5µm) using mobile phase buffer: methanol ph 6.8 (96: 4 v/v) at flow rate of 1.0 ml/min at ambient temperature. The retention times were 9.546 min. for Simvastatin. Calibration plots were linear over the concentration range 1-50µg/ml. Quantification was achieved with photodiode array detection at 254 nm over the concentration range of 1-50 µg/ml.
4. **Stephen Rathinaraj.B**[17] ***et.al*** **(2010)** developed a simple, precise, accurate and rapid high-performance thin-layer chromatographic method and validated for the estimation of Simvastatin and ezetimibe simultaneously in combined dosage forms. The stationary phase used was precoated silica gel 60F 254. The mobile phase used was a mixture of chloroform: benzene: methanol: acetic acid (6.0:3.0:1.0:0.1 v/v/v/v). The detection of spots was carried out at 250 nm. The method was validated. The calibration curve was found to be linear between 0.8 and 4.0 µg/spot for Simvastatin and 0.1 and 1.0 µg/spot

for ezetimibe. The limit of detection and the limit of quantification for Simvastatin were found to be 170 ng/spot and 570 ng/spot respectively, and for ezetimibe, 20 ng/spot and 70 ng/spot respectively.

5. **Varsha Balkrishna Mane**[18] ***et.al*** **(2010)** developed **a** versatile, accurate, precise and economic method for simultaneous determination of Simvastatin and ezetimibe in fixed dose combination products. The absorbance values at 238.2 nm and 247.6 nm and 243.3nm (isoabsorptive point) were used for the estimation of Simvastatin and ezetimibe, respectively without mutual interference. This method obeyed Beer's law in the concentration range of 3–18 μg /ml for Simvastatin and 5-30 μg /ml for ezetimibe. The results of analyses have been validated statistically for linearity, accuracy and precision, LOD and LOQ of the proposed method.
6. **Praveen Kumar S. N**[19]***et.al*** **(2012)** developed a simple, sensitive and validated HPLC method to determine Simvastatin in bulk drug and pharmaceutical formulation. The chromatographic method was achieved on a C18 column (150x4.6 mm, 2.7 μm) using a mixture of methanol and 0.1% ortho phosphoric acid in water (10:90) at a wavelength of 238 nm. The retention time of Simvastatin was found to be 3.106 min. The method was found to show good linearity in the concentration range of 5.0 – 60.0 μg/ml of Simvastatin with correlation co-efficient of 0.9999. Accuracy was between 97.45% and 98.32%.
7. **Nataraj** k.s[20]***et.al*** **(2012)** developed a Reverse phase high performance liquid chromatographic (RP-HPLC) method for the simultaneous estimation of ezetimibe (EZT) and Simvastatin (SMV) in marketed tablet formulation .The determination was carried out on Inertsil ODS-3V, (150 × 4.6 mm, 5μ) column and 0.1% orthophosphoric acid in Milli Q water adjust PH 4.2 with triethylamine, methanol and acetonitrile (45:15:40) used as a mobile phase in gradient technique, at a flow rate of 1.8ml/min, the detection was carried out at 238nm. Retention time of EZT and SMV were found to be 5 and 10.50 min, respectively. The method has been validated according to ICH Q2 (R1) guidelines. Linearity for EZT and SMV were found in the range of 5ppm-15ppm for both drugs. The correlation coefficient (r2) for EZT and SMV were found to be 0.9999 and 0.9998, respectively. The mean recoveries obtained for EZT and SMV were found to be 101% and 101.5%, respectively .Developed method was found to be accurate, precise, selective and rapid for simultaneous estimation of EZT and SMV in tablets.

8. **Effat souri[21]*et.al* (2010)** has developed a rapid and sensitive derivative spectrophotometric method for analysis of these drugs in combined dosage forms. A first order derivative spectrophotometric method was developed for simultaneous determination of Simvastatin and ezetimibe using zero-crossing technique. The measurements were carried out at 219 and 265 nm for Simvastatin and ezetimibe respectively. The described method was found to be linear (r2>0.999) over the range of 2-40 µg/mL for Simvastatin in the presence of 10µg/mL ezetimibe at 219 nm and in the range of 1-20 µg/mL of ezetimibe in the presence of 20 µg/mL of Simvastatin at 265 nm. The within-day and between-day precision values for both drugs were less than 3% (CV).
9. **Madhukar A[22] *et.al* (2012) developed a** analytical method suitable for validation of Simvastatin by reversed Phase High Performance liquid chromatography (RP-HPLC) method. The method utilized RP-HPLC (Younglin HPLC with UV-detector) model and a column, 150mm 4.6 mm, 5m (Symmetry, ODS- 3V, 150mm,4.6mm, 5m). The mobile phases were comprised of Acetonitrile and (0.02M) Buffer pH 3.5 (60:40 v/v). Validation experiments were performed to demonstrate System suitability, precision, linearity and Range, Accuracy study, stability of analytical solution and robustness. The method was linear over the concentration range of 1-150 mg/ML-1. The method showed good recoveries (98.2 – 104.3%).
10. **Dhaneshwar.s.s[23]*et.al* (2008) developed** a simple, precise, and accurate HPTLC method for simultaneous estimation of the compounds as the bulk drugs and in the tablet dosage form. Chromatographic separation was performed on aluminium-backed silica gel 60 F254 plates with 8:2 (*v*/*v*) toluene -2- propanol as mobile phase. The separated spots were densitometrically evaluated at 240 nm. The drugs were satisfactorily resolved with *R*F values 0.48 ± 0.01 and 0.53 ± 0.01for Simvastatin and ezetimibe, respectively. The accuracy and reliability of the method were assessed by determination of validation data for linearity (0.4–2.0 µg per spot for both Simvastatin and ezetimibe), precision (intra-day *RSD* 0.51–1.04%, inter-day *RSD* 0.34–1.11% for Simvastatin; intra-day *RSD* 0.47–0.61%, inter-day *RSD* 0.31–0.61% for ezetimibe), accuracy (98.50 ± 0.23 for Simvastatin and 98.99 ± 0.38 for ezetimibe), and specificity, in accordance with ICH guidelines.

11. **Jyothirmayee.M**[24] ***et.al*** **(2012)** developed a new method based on oxidative coupling of Simvastatin with MBTH (3-Methyl-2-benzothiazolinone hydra zone hydrochloride monohydrate) as a chromogenic derivatizing reagent in presence of Ferric chloride, resulting green color complex with a suitable absorption at 628nm. Optimization studies showed that the coupling reaction is very fast and completed in less than 1 minute. The coupled complex formed exhibits absorption maximum at 628 nm. The assays were linear over the concentration range of 2-8 µg/ml and reproducible. Linear relationship with good correlation coefficients 0.994 were found between absorbance and corresponding concentrations of drug. The reliability and performance of proposed methods was validated statistically the percentage recovery ranged from 100.36-101.75% respectively.
12. **Srinivas .c** [25] ***et.al*** **(2012)** developed a simple, rapid, sensitive, reverse phase isocratic RP-HPLC method for estimation of Simvastatin in bulk and microemulsion formulation. The method was carried out using (Phenomenex Luna C18 5µm 4.6×250mm (i.d) column) with mobile phase comprised of 0.1% Triethylamine buffer (pH 7.5): Acetonitrile (20:80v/v). The flow rate was set at 1.0 ml/min and effluent was detected at 238nm, The retention time of Simvastatin was found to be 8.6 minute. The method developed was validated for specificity, accuracy, precision, linearity and limit of detection, limit of quantification, robustness and stability. The calibration curve was linear in the concentration range of 200-600 ng/ml with correlation coefficient of 0.999. LOD and LOQ were found to be 5ng/ml and 40ng/ml respectively. The percentage recovery for the Simvastatin was found to be 99.15% to 100.53% and the % RSD was found to be 0.980% (i.e <1%).
13. **Mohamed Salim**[26] ***et.al*** **(2012)** developed a novel, quick, reliable and simple capillary zone electrophoresis CZE method and validated for the simultaneous determination of Sitagliptin (SG) and metformin (MF) in pharmaceutical preparations. Separation was carried out in fused silica capillary (50.0 cm total length and 43.0 cm effective length, 49 µm i.d.) by applying a potential of 15 KV (positive polarity) and a running buffer containing 60 mM phosphate buffer at pH 4.0 with UV detection at 203 nm. The samples were injected hydrodynamically for 3 s at 0.5 psi and the temperature of the capillary cartridge was kept at 25 °C. Phenformin was used as internal standard (IS). The method was suitably validated with respect to specificity, linearity, limit of detection and quantization, accuracy, precision, and robustness. The method showed

good linearity in the ranges of 10–100 μg/mL and 50–500 μg/mL with limits of detection of 0.49, 2.11 μg/mL and limits of quantification of 1.48, 6.39μg/mL for SG and MF, respectively.

14. **Bujji Babu**[27] ***et.al*** **(2011)** developed a simple, sensitive and accurate reversed phase high performance liquid chromatographic method for Enoxaparin sodium and Sitaglipitin drugs. Reversed phase chromatographic separation the above two drugs was performed a C18 column is used with different mobile phases of water, orthophosphoric acid, tetrahydrofuranan methanol, acetonitrile, sodiumdihydrogen phosphate, orthophosphoric acid respectively. The detection of wave length is 230 nm for Enoxaparin sodium and 270 nm for Sitagliptin .The percentage of recovery 99.5% for enoxaparin sodium and 97.6% for sitaglipitin. The proposed method is validated for linearity, accuracy, and precision, limit of detection (LOD) and limit of quantification (LOQ) as per the guide lines of Internationational Conference on Harmonization (ICH).
15. **RamziaI**[28] ***et.al*** **(2011)** developed a Simple, accurate and precise SpectroflourImetric and spectrophotometric methods and validated for the determination of Sitagliptin phosphate monohydrate (STG) and metformin HCL (MET). Zero order, first derivative, ratio derivative spectrophotometric methods and flourometric methods have been developed. The zero order spectrophotometric method was used for the determination of STG in the range of 50-300 μg mL−1. The first derivative spectrophotometric method was used for the determination of MET in the range of 2–12 μg mL−1 and STG in the range of 50-300 μg mL−1 by measuring the peak amplitude at 246.5 nm and 275 nm, respectively. The first derivative of ratio spectra spectrophotometric method used the peak amplitudes at 232 nm and 239 nm for the determination of MET in the range of 2–12 μg mL−1. The flourometric method was used for the determination of STG in the range of 0.25-110 μg mL−1.
16. **RamziaI**[29] ***et.al*** **(2011)** developed a Simple, accurate and precise spectrophotometric methods for the determination of Sitagliptin and Vildagliptin in bulk and dosage forms. The proposed methods are based on the charge transferred complexes of Sitagliptin phosphate and Vildagliptin with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), 7,7,8,8-tetracyanoquinodimethane (TC NQ) and tetrachloro-1,4-benzoquinone (*p*-chloranil). All the variables were studied to optimize the reactions conditions. For Sitagliptin, Beer's law was obeyed in the concentration ranges of 50-300 μg/ml, 20-120 μg/ml and 100-900 μg/ml with DDQ, TCNQ and *p*-chloranil, respectively. For

Vildagliptin, Beer's law was obeyed in the concentration ranges of 50-300 µg/ml, 10-85 µg/ ml and 50-350 µg/ml with DDQ, TCNQ and *p*-chloranil, respectively. The developed methods were validated and proved to be specific and accurate for the quality control of the cited drugs in pharmaceutical dosage forms**.**

17. **Bala sekaran.C[30]*et.al* (2010)** developed a simple, sensitive and reproducible spectrophotometric method for the determination of Sitagliptin phosphate in bulk and in pharmaceutical formulations. The proposed method is based on condensation of the primary amino group of Sitagliptin phosphate with acetyl acetone and formaldehyde producing a yellow colored product, which is measured spectrophotometrically at 430nm. The color was stable for about 1 hour. Beer's law is obeyed over a concentration range of 5-25 µg/ml. The apparent molar absorptivity and Sandell sensitivity values are 1.067 x 104 Lmol-1cm-1 and 0.0471 µgcm-2 respectively.
18. **Parag Pathade[31]*et.al* (2011)** developed a simple, sensitive, reproducible and cost effective stability indicating UV Spectrophotometric method for quantitative determination of Sitagliptin Phosphate in bulk and pharmaceutical formulations. The UV spectrum was scanned between 200 to 400 nm and 267 nm was selected as maximum wavelength for absorption. Beer's law was obeyed in the concentration range of 10-100 mg/ml. Good accuracy (99.87-100.45%), precision (%RSD 1.3147-1.2957) were found, the method was successfully applied to the pharmaceutical dosage form containing the above-mentioned drug without any interference by the excipients. The limit of detection and limit of quantification was found to be 0.16µg/ml & 0.45µg/ml respectively.
19. **Jain Pritam[32]*et.al* (2011)** developed a simple, rapid, accurate and economical First order UV-derivative spectrophotometric method for estimation of Sitagliptin from bulk and pharmaceutical formulation. The λmax of Sitagliptin in methanol and water was found to be 267 nm. The same spectrum was derivatised in to first order derivative; showed maximum amplitude of the trough at 275 nm. The drug follows linearity in the concentration range 10-60 µg/ml with correlation coefficient value 0.998. The proposed method was applied to pharmaceutical formulation and % amount of drug estimated 99.19 % was found in good agreement with th label claim. The accuracy of the method was checked by recovery experiment performed at three different levels i.e., 80%, 100% and 120 %. The % recovery was found to be in the range 98.54%– 99.98%. The low values of % R.S.D. are indicative of the accuracy and reproducibility of the

method. The precision of the method was studied as an intra-day, inter-day variations and repeatability. The % R.S.D. value less than 2 indicate that the method is precise. Ruggedness of the proposed method was studied with the help of two analysts.

20. **Sumithra M**[33] ***et.al*** **(2012)** developed a simple, sensitive and rapid reverse phase high performance liquid chromatographic method for simultaneous estimation of Sitagliptin and Metformin. A BDS hypersil C18 column (250x4.0mm,5µ) was used with a mobile phase containing a mixture of phosphate buffer (Ph-4) and Acetonitrile and in the ratio of 60:40. The flow rate was 1.0ml/min and effluents were monitored at 260nm and eluted at 2.8min and 2.0min respectively. Calibration curve was plotted with a range from 2-12µg/ml for Sitagliptin and 20-120 µg/ml for Metformin.
21. **Chellu S. N. Malleswararao** [34] ***et.al*** **(2012)** developed a rapid, specific, accurate and precise reverse phase ultra performance liquid chromatographic (UPLC) method for the simultaneous determination of Sitagliptin phosphate mono-hydrate and Metformin hydrochloride in pharmaceutical dosage forms. The chromatographic separation was achieved on Aquity UPLC BEH C8 100 x 2.1 mm, 1.7 µm, column using a buffer consisting of 10 mM potassium dihydro-gen phosphate and 2 mM hexane-1-sulfonic acid sodium salt (pH adjusted to 5.50 with diluted phosphoric acid) and acetonitrile as organic solvent in a gradient program. The flow rate was 0.2 mL min−1 and the detection wavelength was 210 nm. The limit of detection (LOD) for Sitagliptin phosphate monohydrate and Metformin hydrochloride was 0.2 and 0.06 µg mL−1, respectively. The limit of quantification (LOQ) for Sitagliptin phosphate monohydrate and Metformin hydrochloride was 0.7 and 0.2 µg mL−1, respectively.
22. **Ghazala Khan**[35] ***et.al*** **(2011)** developed a simple, precise and highly selective analytical method for simultaneous estimation of Metformin HCl and Sitagliptin in tablet formulation. Estimation was carried out by multi-component mode of analysis at selected wavelength of 232 nm and 267 nm for Metformin HCl and Sitagliptin respectively in distilled water. The method was found to be linear in the range of 1-40 µg/ml and accuracy of the method was confirmed by recovery studies of tablet dosages forms and was found to be 99.35% and 98.33% for Metformin HCl and Sitagliptin respectively. Initially lab samples were utilized to validate developed method according to ICH guidelines followed by determination of % concentration of Metformin HCl and Sitagliptin in marketed formulation that was found to be 98.26% ± 0.29 and 97.35% ± 1.38 respectively.

23. **Sheetal Sharma[36] *et.al* (2012) developed** two methods are for the determination of Sitagliptin Phosphate and Simvastatin in binary mixture. The first method was based on spectrophotometric determination of two, using simultaneous equation method. It involves absorbance measurement at 267.0 nm (λmax Sitagliptin Phosphate) and 238.0 nm (λmax Simvastatin) in methanol: water in a ratio of 90:10(v/v); linearity was obtained in the range 10-50 µg/ml and 5 –25µg/ml for both the drugs respectively. The second method was based on separation of the two in reverse phase mode using Cosmosil C18 column and The mobile phase consisted Ammonium dihydrogen orthophosphate: ACN (Ph3 with OPA) in the ratio 50:50v/v that was set at a flow rate of1.0ml/min. Linearity was obtained in the concentration range 50-250µg/ml for Sitagliptin and 10-50 µg/ml for Simvastatin. Both these methods were validated according to ICH guidelines and can be successively applied to pharmaceutical formulation.

Chapter-4

Aim & plan of work

Aim and plan of work

Aim of work

The combination dosage form selected for the present study contains Simvastatin and Sitagliptin in solid oral dosage forms, recently this combination has been approved by USFDA (united states food drug administration) in the year 2011.

The aim of work is to develop and validate a simple, precise, accurate and economical RP-HPLC method as per ICH guidelines for the estimation of Simvastatin and Sitagliptin in bulk and pharmaceutical dosage forms.

PLAN OF WORK

- To develop a new RP-HPLC method for the simultaneous estimation of Simvastatin and Sitagliptin.
- To validate the developed method by determining the validation parameters.

Chapter-5

Materials & methods

Materials and methods

1. Reagents and Standard –

Sitagliptin & Simvastatin Tablets were selected with a label of sitagliptin 100mg and simvastatin 20mg manufactured by Merck

a. Water HPLC Grade.

b. Sitagliptin & Simvastatin Working Standards

c. Methanol HPLC Grade

d. Acetonitrite Hplc grade .

e. High performance liquid chromatography equipped with auto sampler and PDA detector.

2. Chromatographic Parameters

Column	-	Agilent C8 (4.6 x 150mm, 3.5µm) or equivalent
Flow rate	-	0.8 mL per min
Wavelength	-	254 nm
Injection volume	-	20 µl
Column oven	-	Ambient
Run time	-	8.0min.
pH	-	6.26.

Preparation of Phosphate buffer

Weighed 2.950 grams of potassium dihydrogen phosphate and 540mg of dipotassium hydrogen ortho phosphate into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, the pH was found to be 6.26.

Preparation of mobile phase

Mixed the above buffer 250 mL (25%) and 750 mL of Acetonitrile HPLC (75%) and degassed in ultrasonic water bath for 5 minutes. Filtered through 0.45 µ under vacuum filtration.

Diluent preparation

Mixed the above buffer 250 mL (25%) and 750 mL of Acetonitrile HPLC (75%) and degassed in ultrasonic water bath for 5 minutes. Filtered through 0.45 µ under vacum filtration.

Standard Solution Preparation

Accurately weighed and transferred 10 mg each of Sitagliptin & Simvastatin working standard into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent (Stock solution).Further pipetted 1ml of Sitagliptin & 0.2ml of Simvastatin the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluents.

Sample Solution Preparation

Accurately weighed and transferred equivalent to 10 mg of Sitagliptin/ Simvastatin sample into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent (Stock solution). Further pipetted 1.0ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with Diluent.

TRAIL-1

Preparation of mobile phase

The mobile phase was prepared by mixing 0.1M ammonium acetate buffer and Acetonitrile in the ratio 20:80. The mobile phase is then sonicated using Ultra-Sonicator to remove the impurities and dissolved gases, as they may lead to unwanted peaks in the chromatogram.

Mobile phase: 0.1M Ammonium acetate buffer: Acetonitrile (20:80)

Chromatographic Parameters

Equipment	:	HPLC Shimadzu Separation Module LC-20AT Prominence Liquid Chromatograph
Column	:	Hypersil BDS 250×4.6, 5µm column
Flow rate	:	1.0ml/min
Wavelength	:	254nm
Injection volume	:	20 µl

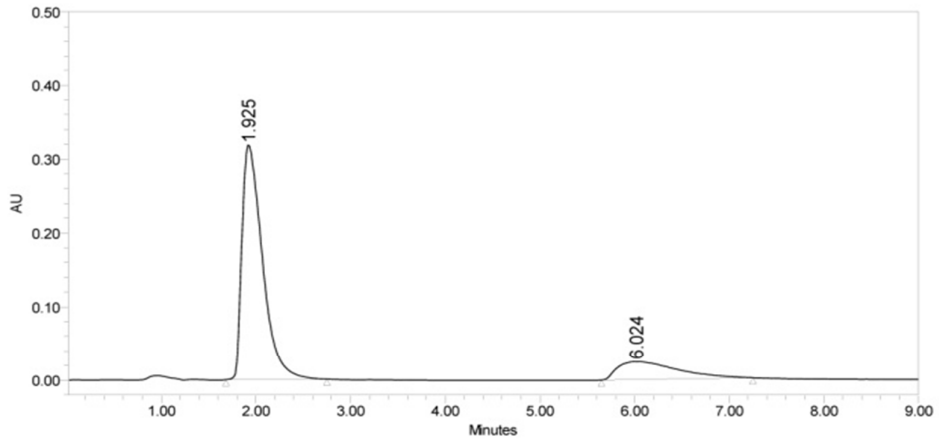
Column oven	:	Ambient
Run time	:	9min

Preparation of mobile phase

The mobile phase was prepared by mixing 0.1M ammonium acetate buffer and Acetonitrile in the ratio 35:65. The mobile phase is then sonicated using Ultra-Sonicator to remove the impurities and dissolved gases, as they may lead to unwanted peaks in the chromatogram.

SAMPLE INFORMATION

Sample Name:	SITA_SIM	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	SITA_SIM
Vial:	80	Acq. Method Set:	Prashanti sit_sim
Injection #:	1	Processing Method:	Sita_Sim Trail 3
Injection Volume:	20.00	Channel Name:	2487Channel 1
Run Time:	10.0 Minutes	Proc. Chnl. Descr.:	220



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Simvastatin	1.925	4803376	319933	378.6	2.0	
2	Sitagliptin	6.024	1070934	24384	436.2	2.1	5.24

Chromatogram-1

Conclusion

The peak shapes are not clear and the retention time of simvastatin was also very low.

TRAIL-2

Preparation of Mobile phase

The mobile phase is prepared by mixing sodium acetate buffer (pH-3) and methanol in the ratio of 45:55. The mobile phase is then sonicated using Ultra-Sonicator to remove the impurities and dissolved gases, as they may lead to unwanted peaks in the chromatogram.

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Mobile phase: Sodium acetate buffer: methanol (45:55)

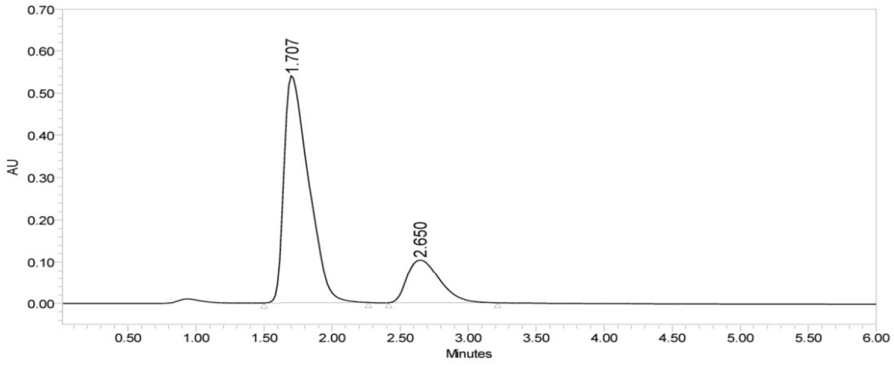
Chromatographic Parameters

Equipment	:	High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector
Column	:	Xterra 4.6×150 mm column
Flow rate	:	1.0ml/min
Wavelength	:	254nm
Injection volume	:	20 µl
Column oven	:	Ambient
Run time	:	6min

Trail-2

SAMPLE INFORMATION

Sample Name:	SITA_SIM	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	SITA_SIM
Vial:	80	Acq. Method Set:	Prashanti sit_sim
Injection #:	2	Processing Method:	Sita_Sim trail 5
Injection Volume:	20.00	Channel Name:	2487Channel 1
Run Time:	15.0 Minutes	Proc. Chnl. Descr.:	220



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Simvastatin	1.707	6795530	543170	376.9	1.7	
2	Sitagliptin	2.650	1721351	103414	597.4	1.5	2.36

Chromatogram-2

Conclusion

The retention time was very low for simvastatin which has been tried for many concentrations.

TRAIL-3

Preparation of Phosphate buffer

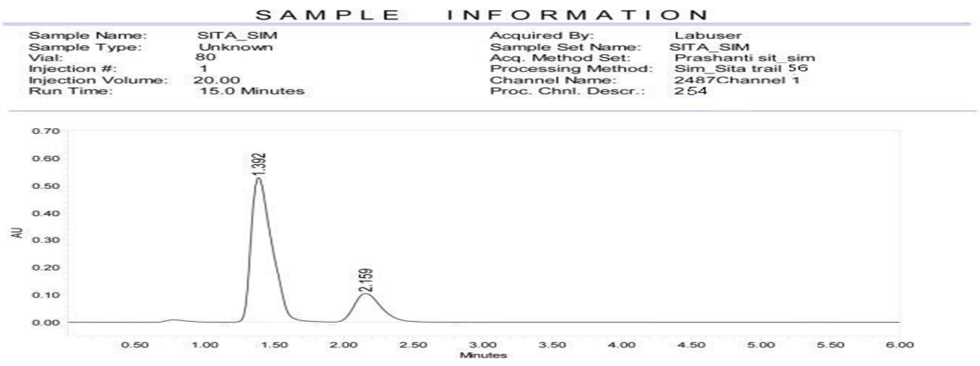
Weighed 2.950 grams of potassium dihydrogen phosphate and 540mg of dipotassium hydrogen ortho phosphate into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, the pH was found to be 6.26.

The mobile phase is prepared by mixing Phosphate buffer (pH-6.26) and Acetonitrile in the ratio of 50:50. The mobile phase is then sonicated using Ultra-Sonicator to remove the impurities and dissolved gases, as they may lead to unwanted peaks in the chromatogram.

Mobile phase: Phosphate buffer: methanol (50:50)

Chromatographic Parameters

Equipment	:	High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector
Column	:	Xterra 4.6×150 mm column
Flow rate	:	1.0ml/min
Wavelength	:	254nm
Injection volume	:	20 µl
Column oven	:	Ambient.
Run time	:	6min



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Simvastatin	1.392	5462650	532594	376.8	1.5	
2	Sitagliptin	2.159	1404771	103633	603.2	1.3	2.34

Chromatogram-3

Conclusion

The usp plate count was found to be less and the retention time was found to be less for sitagliptin with an less tailing which was found unsatisfactory**.**

TRAIL-4

Preparation of Phosphate buffer

Weighed 2.950 grams of potassium dihydrogen phosphate and 540mg of dipotassium hydrogen ortho phosphate into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, the pH was found to be 6.26.

The mobile phase is prepared by mixing Phosphate buffer (pH-6.26) and Acetonitrile in the ratio of 40:60. The mobile phase is then sonicated using Ultra-Sonicator to remove the impurities and dissolved gases, as they may lead to unwanted peaks in the chromatogram.

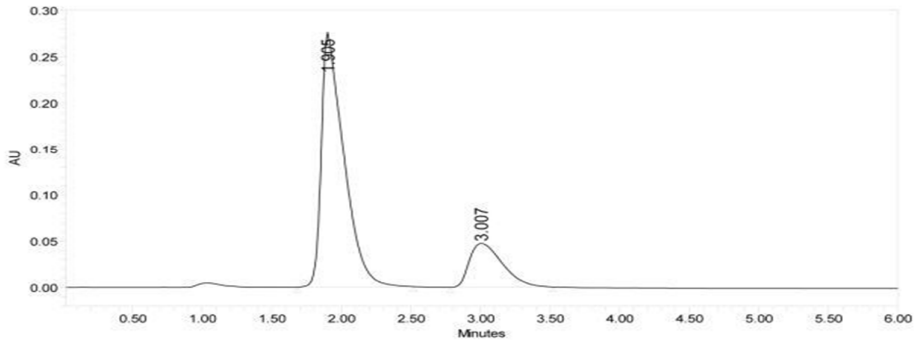
Mobile phase: Phosphate buffer: methanol (40:60)

Chromatographic Parameters

Equipment	:	High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector
Column	:	BDS 4.6×250 mm column
Flow rate	:	0.9ml/min
Wavelength	:	254nm
Injection volume	:	20 µl
Column oven	:	Ambient
Run time	:	6min

SAMPLE INFORMATION

Sample Name:	SITA_SIM	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	SITA_SIM
Vial:	8	Acq. Method Set:	Prashanti sit_sim
Injection #:	1	Processing Method:	Sim_Sita trail
Injection Volume:	20.00	Channel Name:	2487Channel 1
Run Time:	15.0 Minutes	Proc. Chnl. Descr.:	254



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Simvastatin	1.905	3092578	276906	596.2	1.9	
2	Sitagliptin	3.007	779311	47320	757.5	1.6	2.92

Chromatogram-4

Conclusion

The small hump was seen in chromatogram with less tailing and plate count which was carried for many times hence found unsatisfactory**.**

TRAIL-5

Preparation of Phosphate buffer

Weighed 2.950 grams of potassium dihydrogen phosphate and 540mg of dipotassium hydrogen ortho phosphate into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, the pH was found to be 6.26.

The mobile phase is prepared by mixing Phosphate buffer (pH-6.26) and Acetonitrile in the ratio of 30:70. The mobile phase is then sonicated using Ultra-Sonicator to remove the impurities and dissolved gases, as they may lead to unwanted peaks in the chromatogram.

Mobile phase: Phosphate buffer: methanol (30:70)

Chromatographic Parameters

Equipment : High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector

Column : Waters 5.6×150 mm column

Flow rate : 1.0ml/min

Wavelength : 254nm

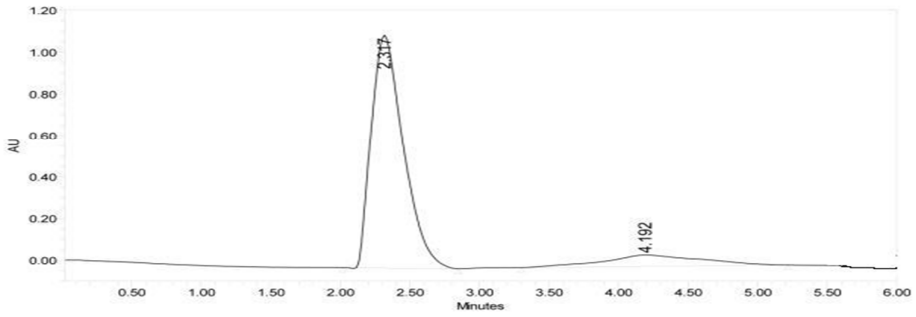
Injection volume : 20 µl

Column oven : Ambient

Run time : 6min

SAMPLE INFORMATION

Sample Name:	SITA_SIM	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	SITA_SIM
Vial:	80	Acq. Method Set:	Prashanti sit_sim
Injection #:	2	Processing Method:	Sim_Sita trail
Injection Volume:	20.00	Channel Name:	2487Channel 1
Run Time:	10.0 Minutes	Proc. Chnl. Descr.:	254



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	USP Resolution
1	Simvastatin	2.317	17947066	1119055	414.2	1.5	
2	Sitagliptin	4.192	2682919	55058	108.3	1.0	1.88

Chromatogram-5

Conclusion

The peak shape was not clear for simvastatin resolution was found to be less with less plate count hence tried by changing the concentration which gave rise to the optimized method.

OPTIMISED METHOD

The objective of this experiment was to optimize the assay method for estimation of Simvastatin and Sitagliptin based on the literature survey .So here the trials mentioned describes how the optimization was done.

Preparation of buffer

Mixture of above buffer 250 ml (25%) and 750 mL of Acetonitrile HPLC (75%) are mixed and degassed in ultrasonic water bath for 5 minutes. Filtered through 0.45 µ under vacuum filtration. The mobile phase is sonicated using Ultra-Sonicator for 5min to remove the impurities and dissolved gases, as they may lead to unwanted peaks in the chromatogram.

Mobile phase: Mixed phosphate buffer: Acetonitrile (25:75).

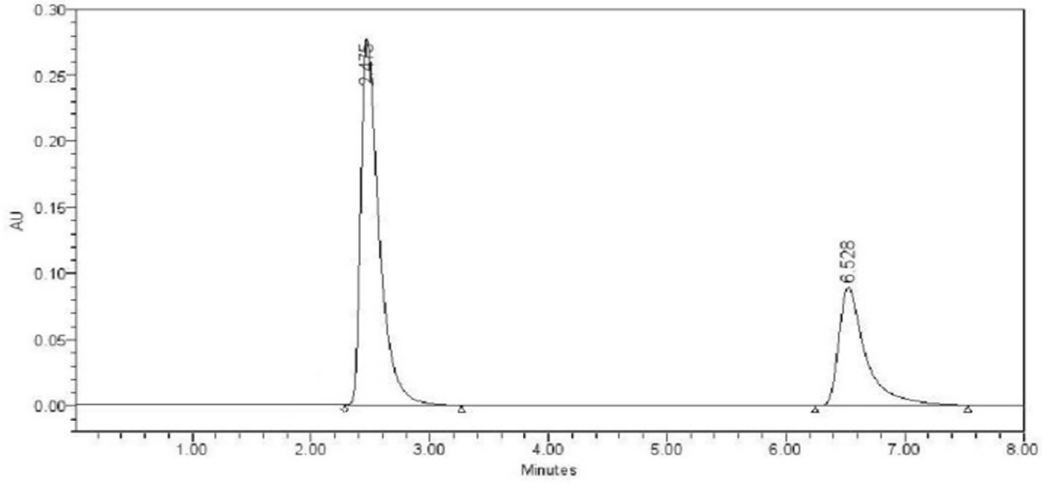
Chromatographic Parameters

Equipment: High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector

Column	-	Agilent C8 (4.6 x 150mm, 3.5µm) or equivalent
Flow rate	-	0.8 ml per min
Wavelength	-	254 nm
Injection volume	-	20 µl
Column oven	-	Ambient
Run time	-	8.0min
PH	-	6.26.

METHOD DEVELOPMENT

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simvaMethod develop
Injection #:	1	Processing Method:	S_S Method development
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.475	2975090	280212	2326.67		1.13
2	Simvastatin	6.528	1417247	89515	4810.76	11.99	1.19

CHROMATOGRAM-6

Conclusion

The retention time, shape, resolution were found to be good when compared to other methods in trails, hence this method was finalized for the estimation of Simvastatin and Sitagliptin. A simple and sensitive reverse phase HPLC method has been developed for simultaneous analysis of Simvastatin and Sitagliptin in combined dosage form. The method utilizes sample preparation followed by separation on a Agilent C8 (4.6 x 150mm, 3.5µm).

Analytes were monitored by UV detection at 254nm using an isocratic mode with Mixed phosphate buffer (ph-6.26): Acetonitrile in the ratio 25:75 as mobile phase. The flow rate was set at 0.8 ml/min and effluent was monitored at 254nm. The retention time was 2.475min and 6.528min for Simvastatin and Sitagliptin respectively.

METHOD VALIDATION

System suitability

Preparation of Phosphate buffer

Weighed 2.950 grams of potassium dihydrogen phosphate and 540mg of dipotassium hydrogen ortho phosphate into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water and the PH was found to be 6.26.

Preparation of mobile phase

Mixture of above buffer 250 ml (25%) and 750 ml of Acetonitrile HPLC (75%) are mixed and degassed in ultrasonic water bath for 5 minutes. Filtered through 0.45 µ filtered under vacuum filtration.

Diluent preparation

Mixture of above buffer 250 ml (25%) and 750 ml of Acetonitrile HPLC grade (75%) are mixed and degassed in ultrasonic water bath for 5 minutes. Filtered through 0.45 µ filtered under vacuum filtration.

Standard solution Preparation

Accurately weighed and transferred 10 mg each of Sitagliptin & Simvastatin working standard into a 10ml clean dry volumetric flask added about 7ml of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution).Further pipetted 1ml of Sitagliptin & 0.2ml of Simvastatin the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluents.

Sample Solution Preparation

Accurately weighed and transferred equivalent to 10 mg of Sitagliptin& 10mg of Simvastatin sample into a 10ml clean dry volumetric flask added about 7ml of Diluent and sonicated it for 5min to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution). Further pipetted 1.0ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with Diluent.

System suitability parameters are integral part of liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The key to achieving system suitability is an optimal instrumentation and an efficient analytical column. System suitability test is a pharmacopoeial requirement. A few commonly used system suitability parameters are given below.

Theoretical plates (N)

It is measure of column efficiency. A column can be considered as being made of large number of theoretical plates where distribution of sample between liquid - liquid or solid - liquid phase occurs. The number of theoretical plates in column is given by the relationship.

$$N = 16 (tR/W)2$$

(OR)

$$N = 5.54 (tR/W1/2)2 \text{ ----- } (1)$$

Where,

tR = Retention time

W = width at the base of the peak.

N should be > 2000.

Retention time (Rt)

Retention time is the time required for elution of the peak maximum after injection of compound.

Resolution (Rs)

It is a function of column efficiency and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system. For the separation of two components in mixture, the resolution is determined by equation.

$$Rs = 2 (tR2 – t R1) / (W1+W2) \text{ ----- } (2)$$

Where,

d t1 = Retention times of second and first compounds respectively

W2 and W1 = Corresponding widths at the bases of peak obtained by

extrapolating straight sides of the peaks to baselines.

Tailing factor (T)

It is the measure of peak symmetry and is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

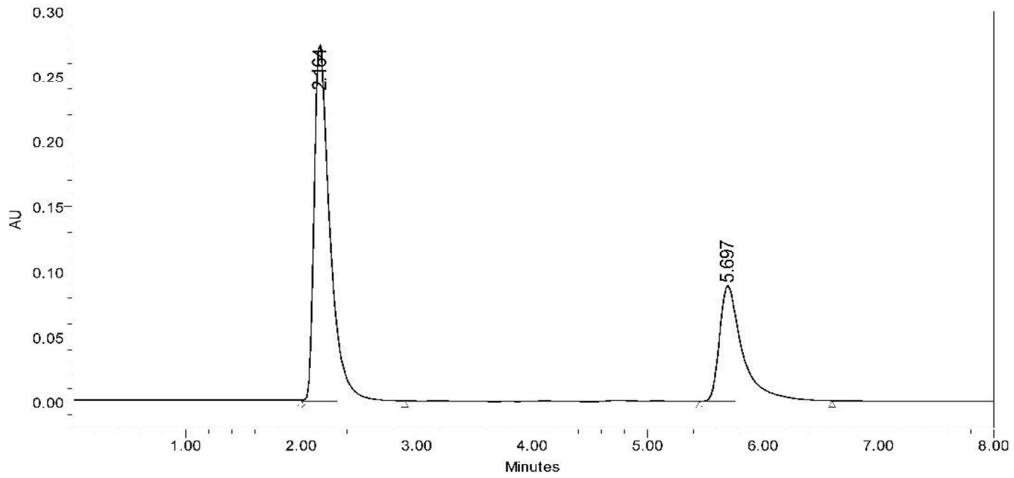
$$T = W\ 0.05 / 2\ f \text{ ----- (3)}$$

Where, W0.05 =width of peak at 5 % height

f = distance from the peak maximum to the leading edge of the peak height from the baseline.

System suitability-1

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknow n	Sample Set Name:	Sita_simva Sample set
Vial:	90	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S System suitability
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

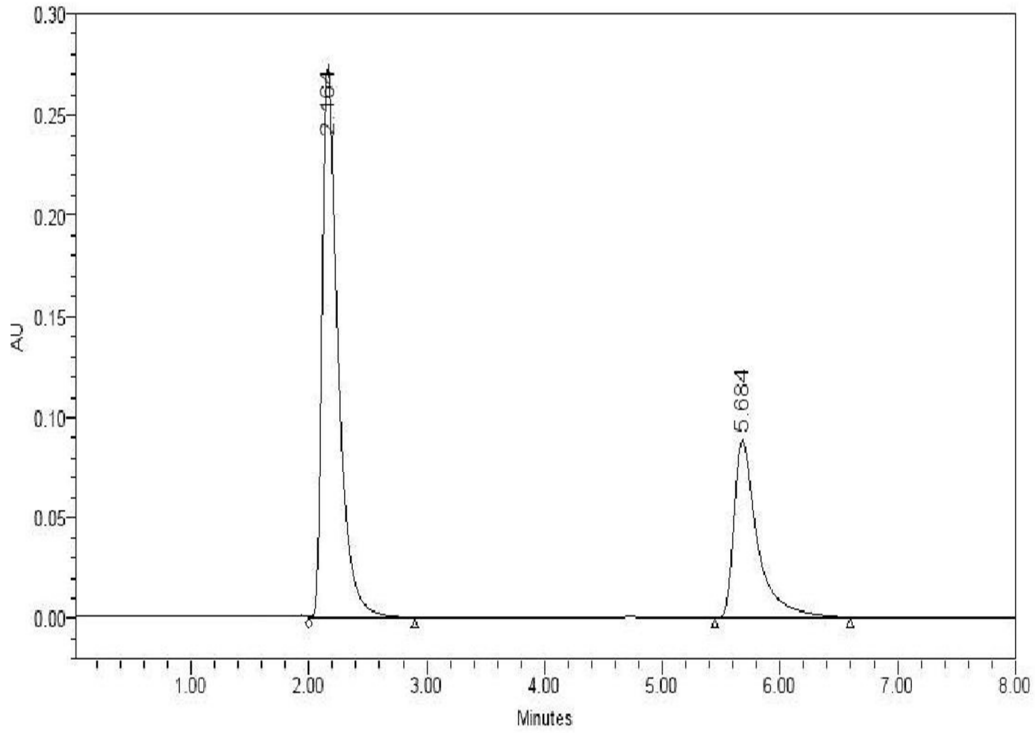


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.164	2596332	274179	2295.21		1.18
2	Simvastatin	5.697	1233290	89010	4767.59	11.89	1.15

Chromatogram-7

System suitability-2

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	92	Acq. Method Set:	sita_simva
Injection #:	2	Processing Method:	S_S System suitability
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

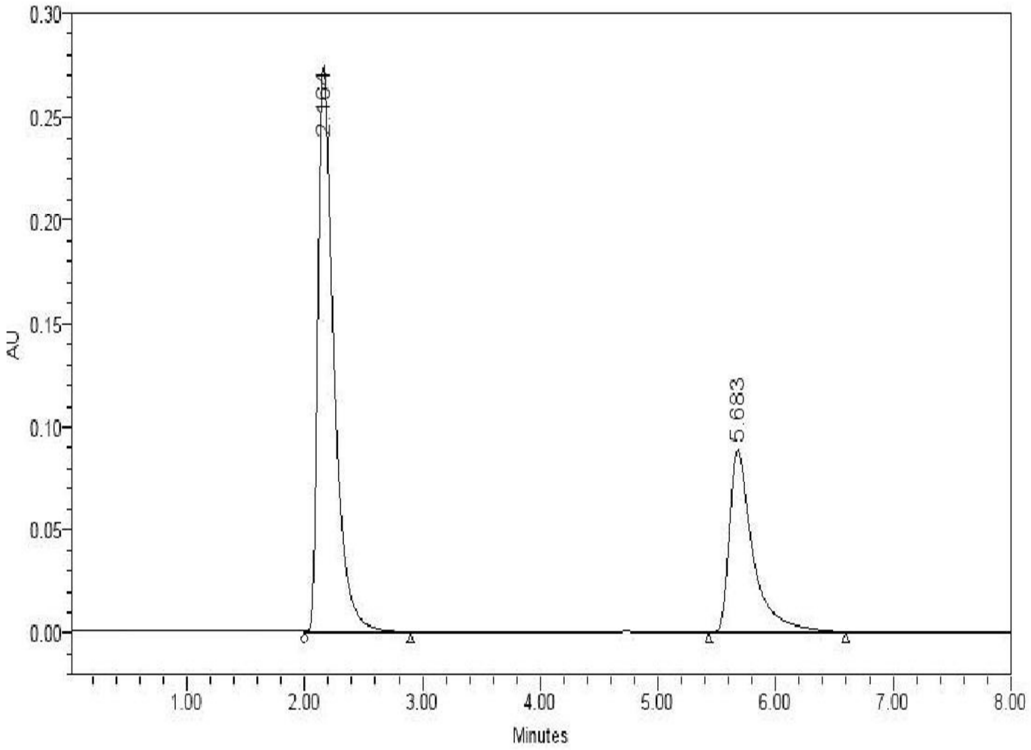


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.164	2591389	274450	2280.41		1.16
2	Simvastatin	5.684	1237254	89198	4751.62	11.81	1.15

Cromatogram-8

System suitability-3

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	98	Acq. Method Set:	sita_simva
Injection #:	3	Processing Method:	S_S System suitability
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

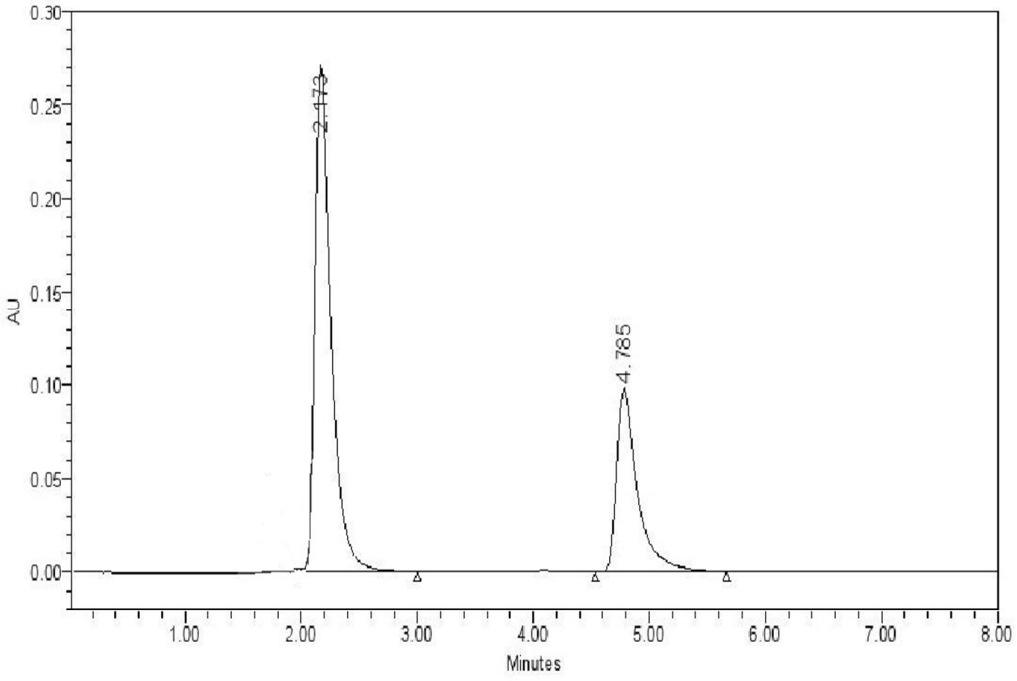


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.164	2602421	274787	2289.07		1.17
2	Simvastatin	5.683	1242461	89396	4728.27	11.80	1.16

Chromatogram-9

System suitability-4

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simva System suitability
Injection #:	1	Processing Method:	S_S MORE ORG
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

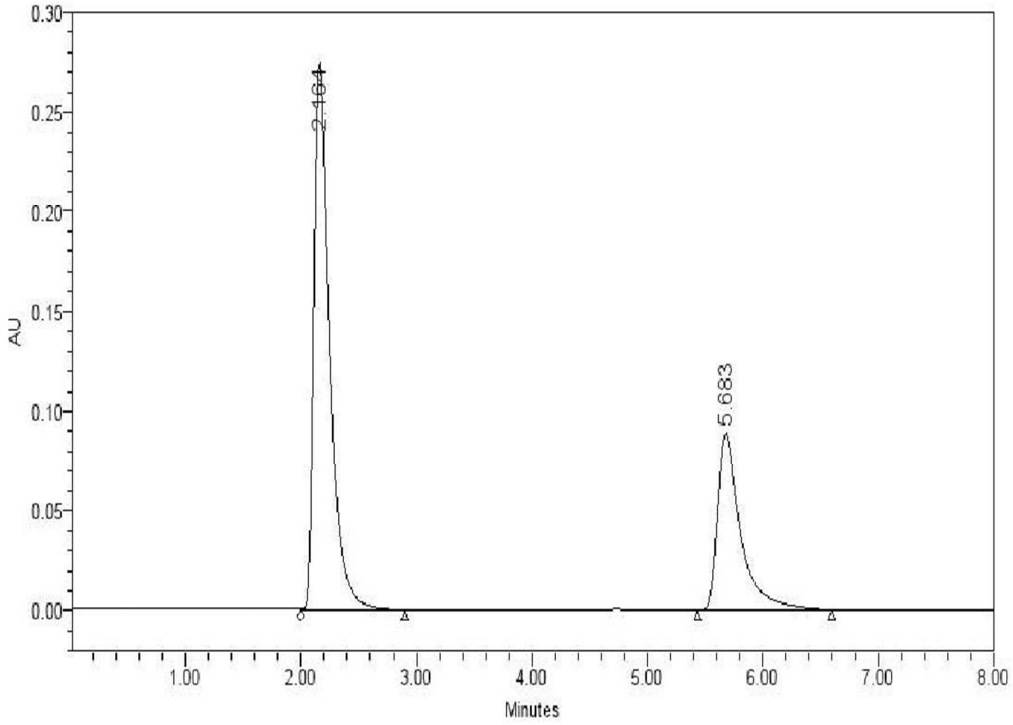


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.173	2644123	272443	224553		1.68
2	Simvastatin	4.785	1229838	98152	3986.48	9.05	1.93

Chromatogram-10

System suitability-5

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	98	Acq. Method Set:	sita_simva
Injection #:	3	Processing Method:	S_S System suitability
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.164	2602421	274787	2289.07		1.17
2	Simvastatin	5.683	1242461	89396	4728.27	11.80	1.16

Chromatogram-11

Data of system suitability for Sitagliptin and Simvastatin

Serial number	Parameter	Sitagliptin	Simvastatin	Acceptance criteria
1	Theoretical plates	2286.158	4608.944	Theoretical plate count should be more than 2000.
2	Retention time	2.232	5.6754	Retention time should be more than 2.
3	Resolution	11.27	11.27	Resolution between two peaks must be more than 2
4	Tailing factor	1.264	1.316	Tailing factor must be less than 2.

Table-3

SPECIFICITY

Standard Solution Preparation

Accurately weighed and transferred 10 mg each of Sitagliptin & Simvastatin working standard into a 10mL clean dry volumetric flask added about 7mL of diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Further pipetted 1ml of Sitagliptin & 0.2ml of Simvastatin the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluents.

Sample Solution Preparation

Accurately weighed and transferred equivalent to 10 mg of Sitagliptin / Simvastatin sample into a 10mL clean dry volumetric flask added about 7mL of diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution).

Further pipetted 1.0ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of Placebo solution

Accurately weighed a quantity of placebo powder 87.6mg of placebo in a 50ml volumetric flask added 30ml of diluent and made upto volume with diluent. Filtered using 0.45 micron membrane filtered. Diluted 5ml to 10ml with diluent.

Preparation of Placebo+ Standard solution

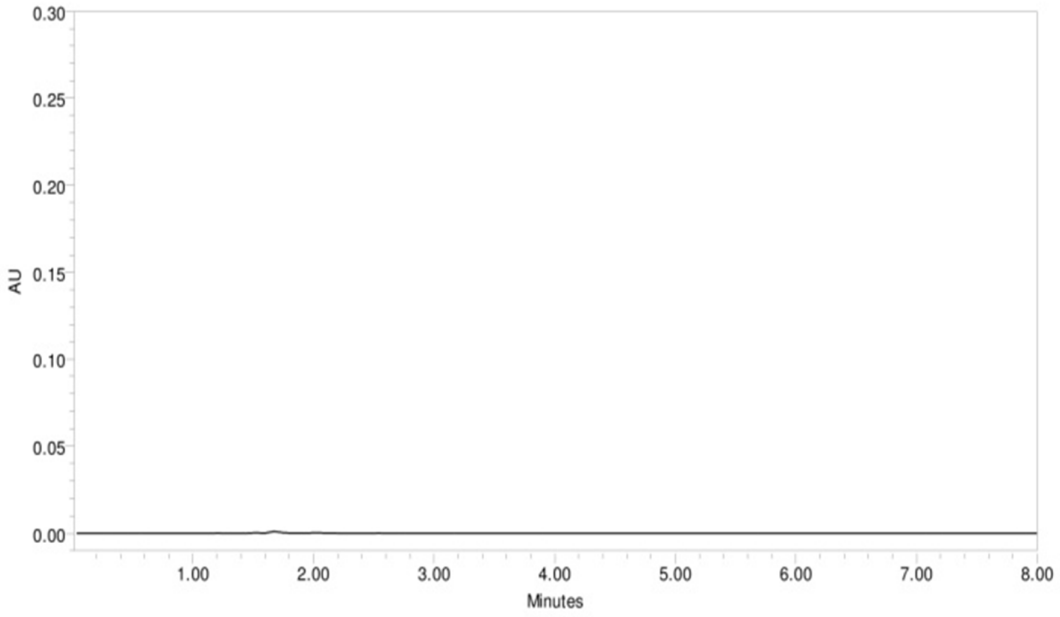
Diluted 5ml of the standard stock solution into 10ml volumetric flask made up the volume with the placebo solution.

Acceptance criteria

There should not be any interference due to placebo in sample and standard preparation.

SPECIFICITY-BLANK

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	9	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	Blank
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

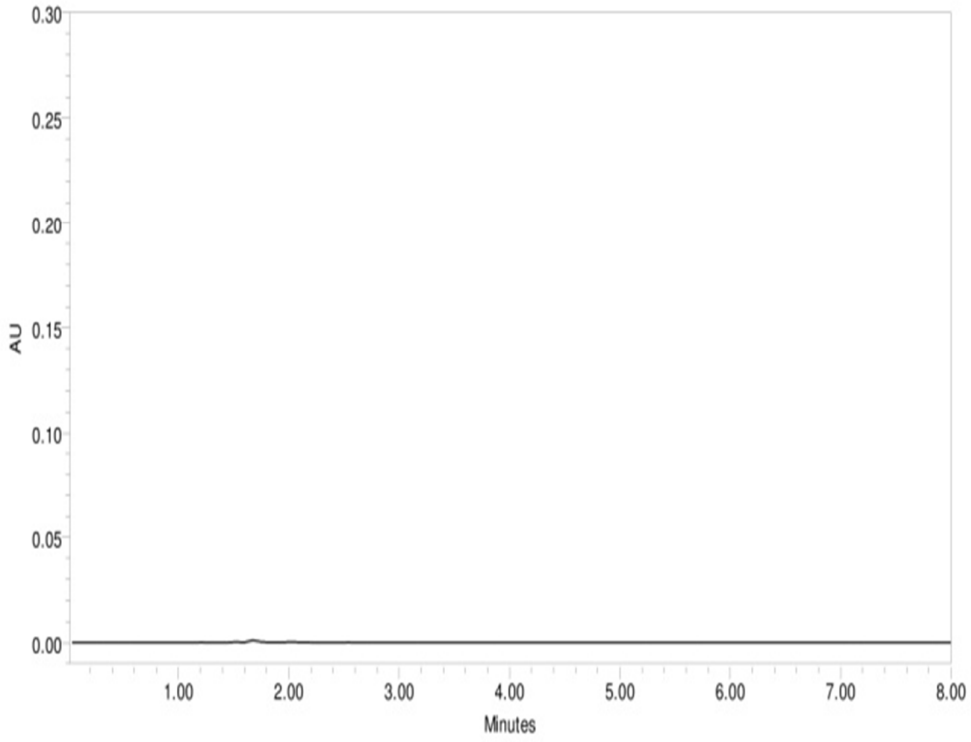


Serial number	Retention time	Peak area	Tailing factor	Number of theoretical plates
NO PEAKS WERE FOUND				

Chromatogram-12

SPECIFICITY-PLACEBO

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	9	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	Placebo
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

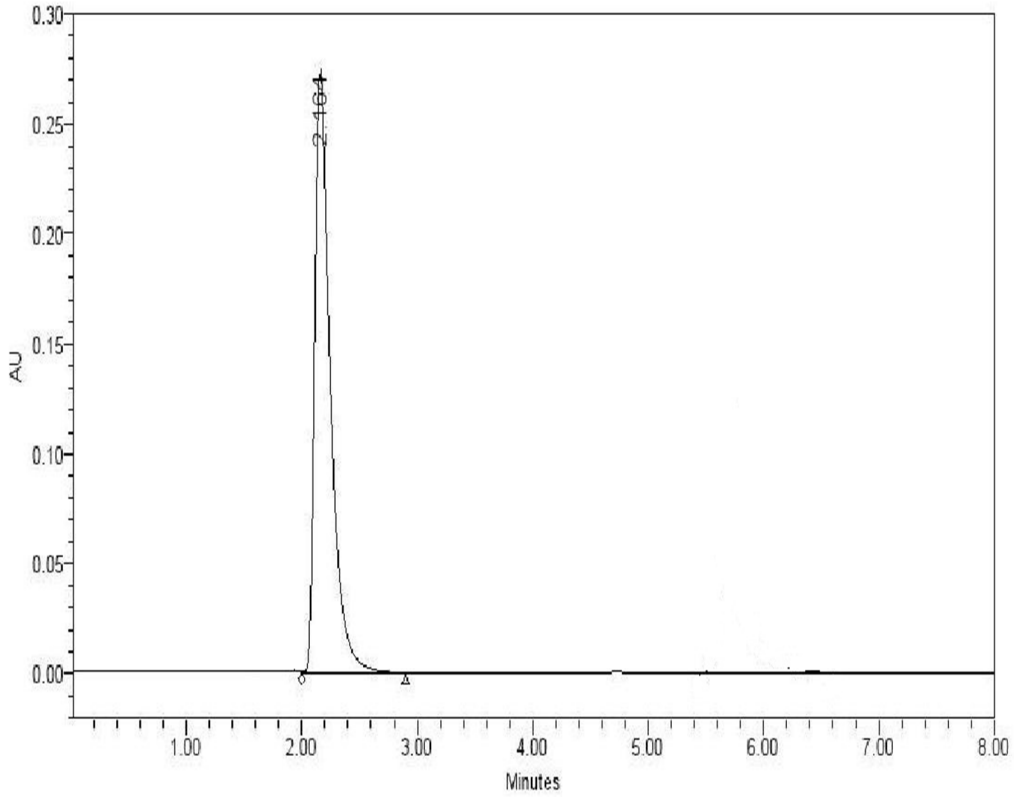


Serial number	Retention time	Peak area	Tailing factor	Number of theoretical plates
NO PEAKS WERE FOUND				

Chromatogram-13

SPECIFICITY-SITAGLIPTIN STANDARD

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	16	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S STD
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

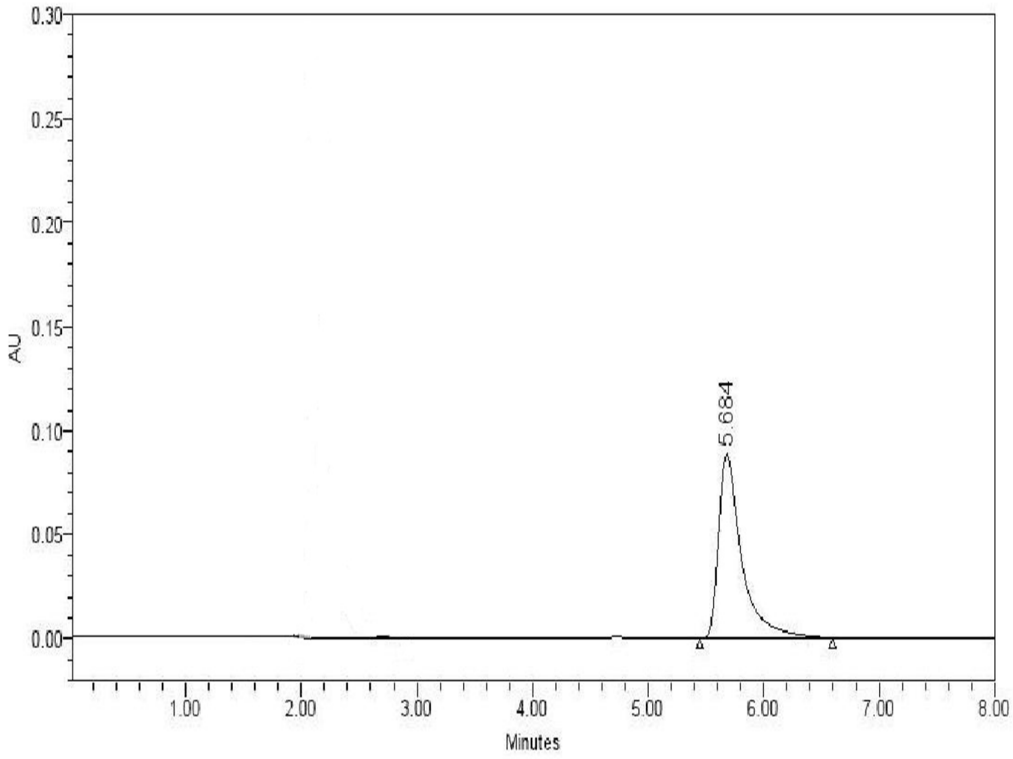


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.164	2591383	274450	2280.41		1.16

Chromatogram-14

SPECIFICITY- SIMVASTATIN STANDARD

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	17	Acq. Method Set:	sita_simva
Injection #:	2	Processing Method:	S_S STD
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

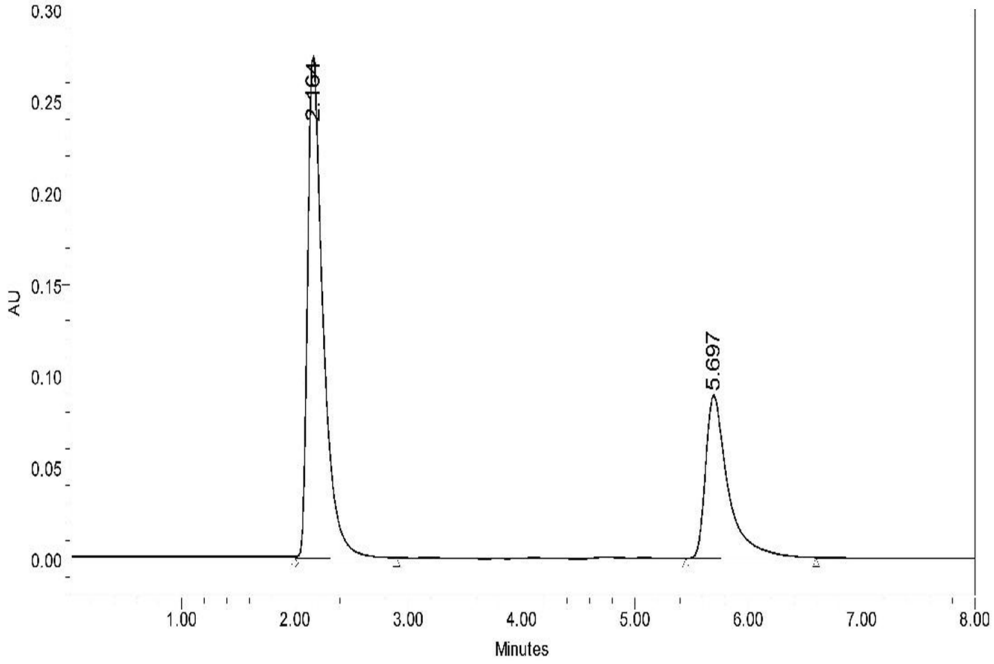


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Simvastatin	5.684	1237254	89198	4751.62		1.15

Chromatogram-15

SPECIFICITY-MIXED STANDARD

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	98	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S STD
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

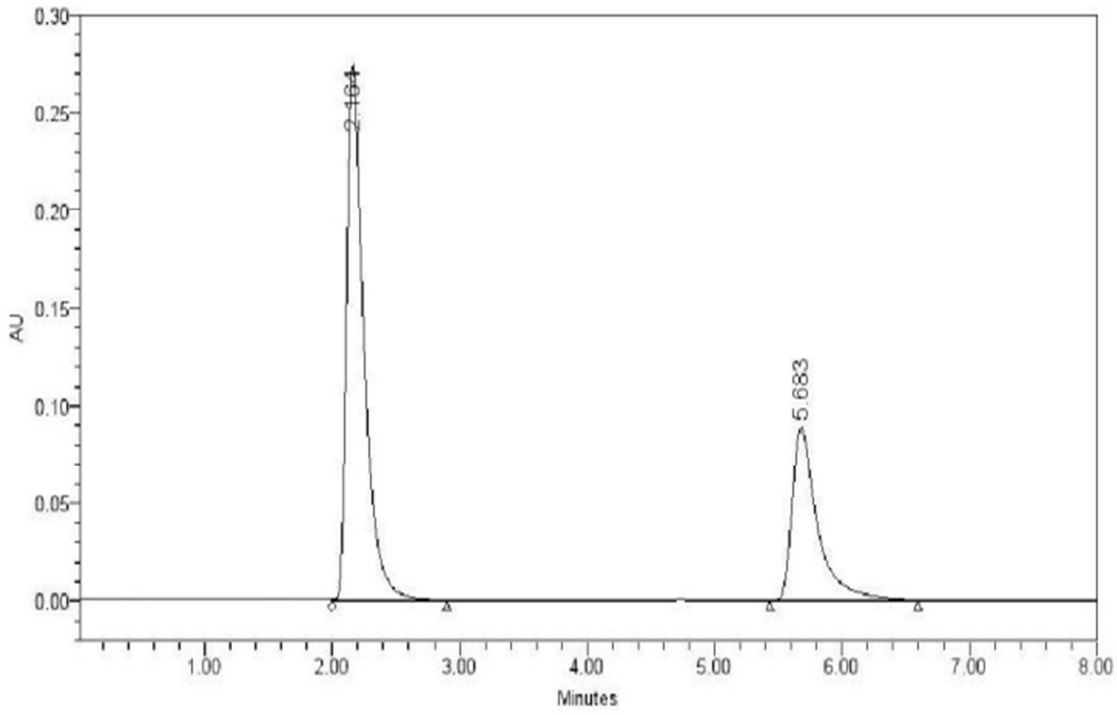


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.164	2596332	274179	2295.21		1.18
2	Simvastatin	5.697	1233290	89010	4767.59	11.89	1.15

Chromatogram-16

SPECIFICITY-PLACEBO+STANDARD

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	98	Acq. Method Set:	sita_simva
Injection #:	3	Processing Method:	S_S System suitability
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

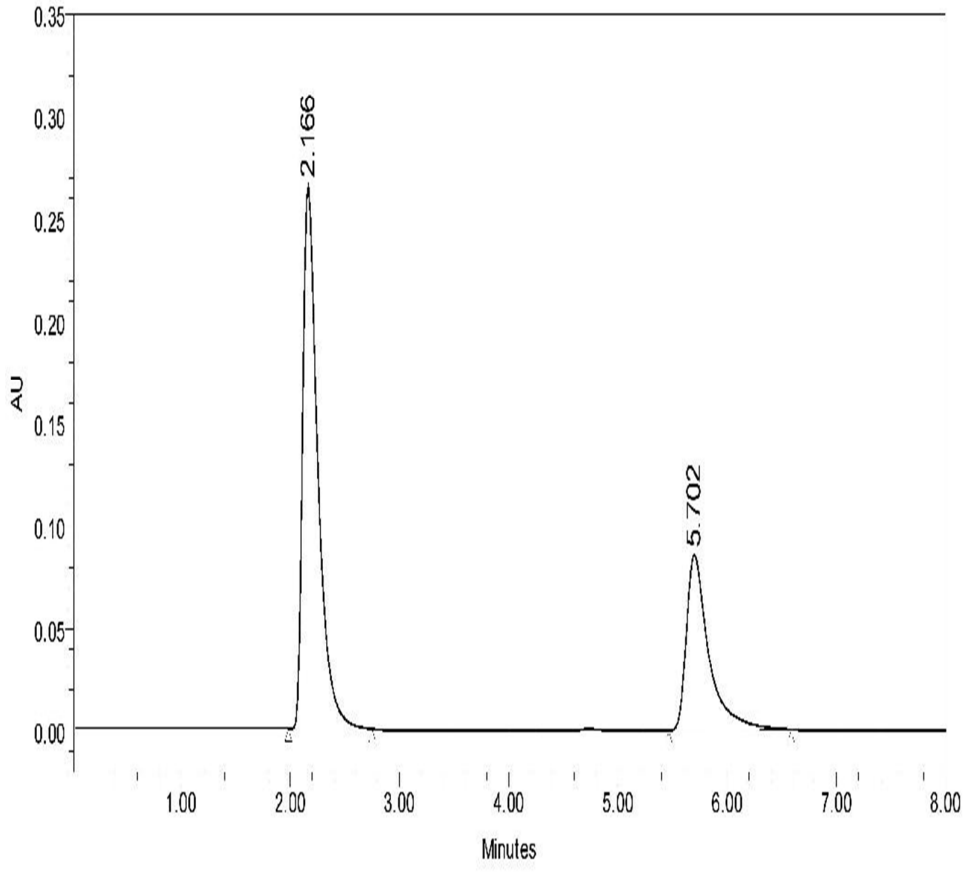


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.164	2602421	274787	2289.07		1.17
2	Simvastatin	5.683	1242461	89396	4728.27	11.80	1.16

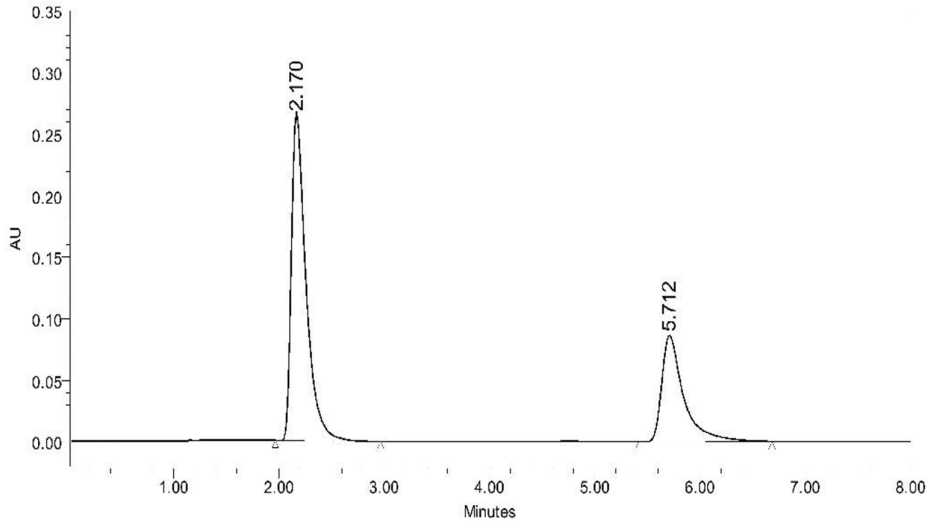
Chromatogram-17

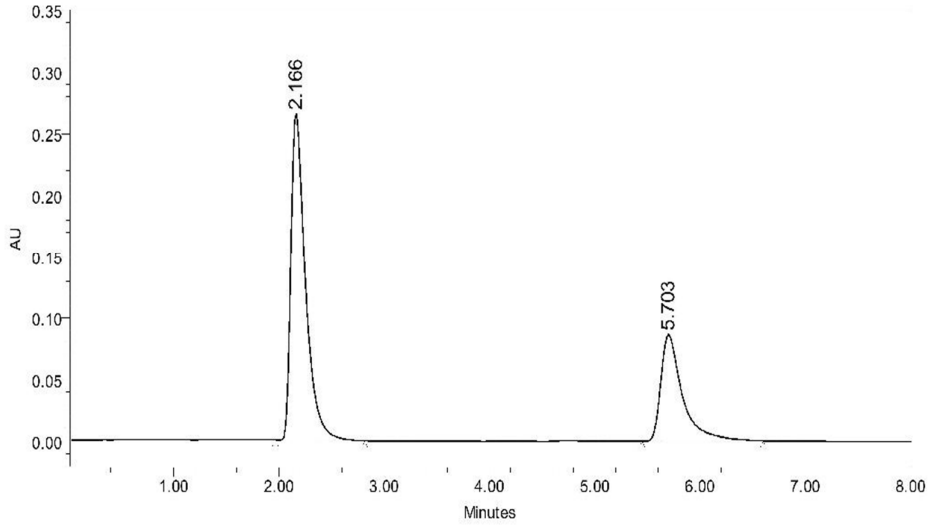
SPECIFICITY-SAMPLE SOLUTION

Sample Name:	SIM SITA Sample	Acquired By:	lab user
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simva
Injection #:	1, 2, 3	Processing Method:	S_S SAMPLE
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



Chromatogram-18

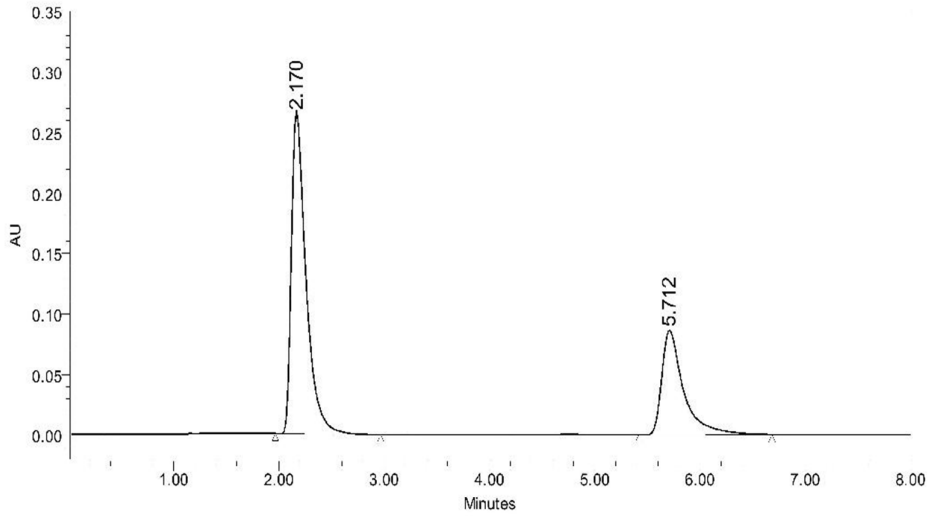


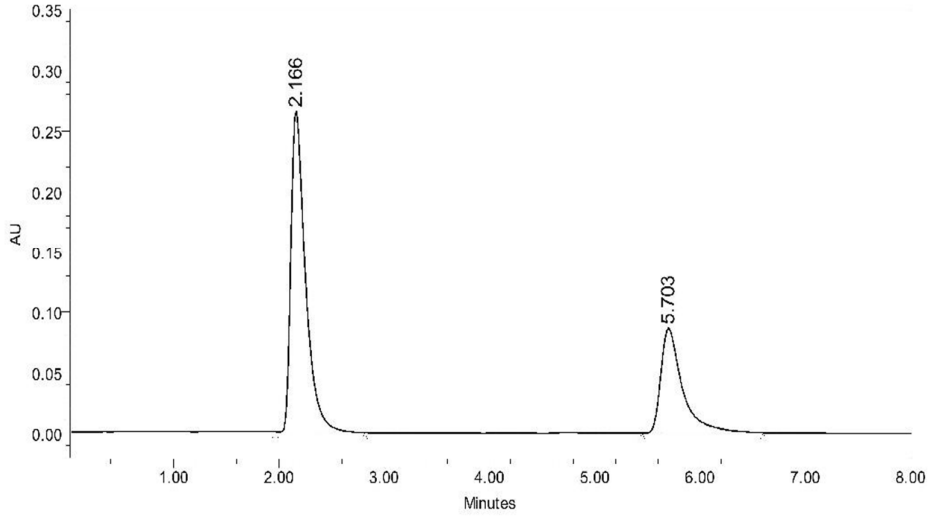


	Name	RT	Area	Height (µV)
1	Sitagliptin	2.166	2547475	265218
2	Simvastatin	5.703	1229732	86392
3	Sitagliptin	2.166	2557852	265752
4	Simvastatin	5.702	1230529	86178

	Name	RT	Area	Height (µV)
5	Sitagliptin	2.170	2565076	267064
6	Simvastatin	5.712	1242227	86409

Chromatogram-19&20





	Name	RT	Area	Height (μV)
1	Sitagliptin	2.166	2547475	265218
2	Simvastatin	5.703	1229732	86392
3	Sitagliptin	2.166	2557852	265752
4	Simvastatin	5.702	1230529	86178

	Name	RT	Area	Height (μV)
5	Sitagliptin	2.170	2565076	267064
6	Simvastatin	5.712	1242227	86409

Chromatogram 21&22

LINEARITY

Preparation of stock solution

Accurately weighed and transferred 10 mg of Sitagliptin and Simvastatin working standard into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (80ppm of Sitagliptin & 16ppm of Simvastatin)

0.8ml of stock solution has taken in 10ml of volumetric flask diluted up to the mark wit diluent.

Preparation of Level – II (90ppm of Sitagliptin & 18ppm of Simvastatin)

0.9ml of stock solution has taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – III (100ppm of Sitagliptin & 20ppm of Simvastatin)

1.0ml of stock solution has taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – IV (110ppm of Sitagliptin & 22ppm of Simvastatin)

1.1ml of stock solution has taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – V (120ppm of Sitagliptin & 24ppm of Simvastatin)

1.2ml of stock solution has taken in 10ml of volumetric flask diluted up to the mark with diluent.

Procedure

Inject each level into the chromatographic system and measure the peak area.

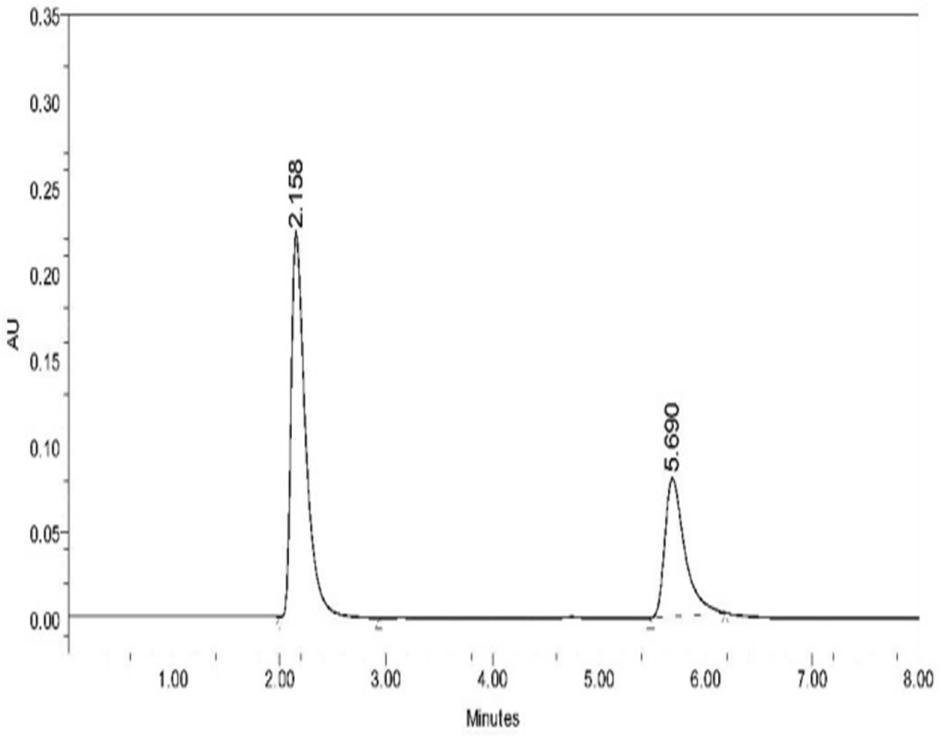
Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Acceptance criteria

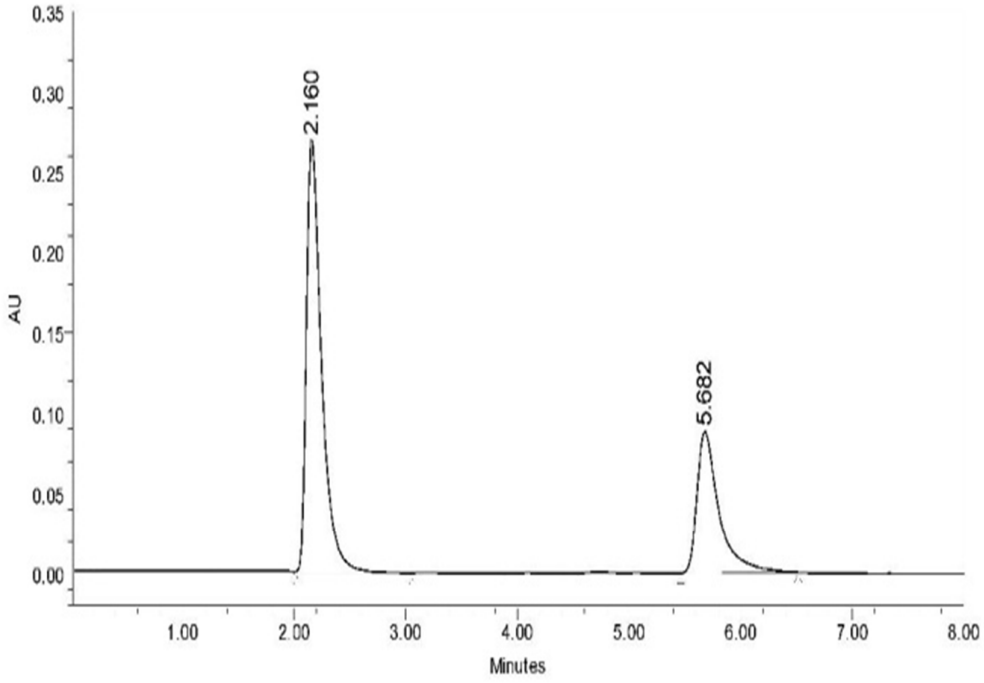
The Linear Regression coefficient should not be less than 0.995.

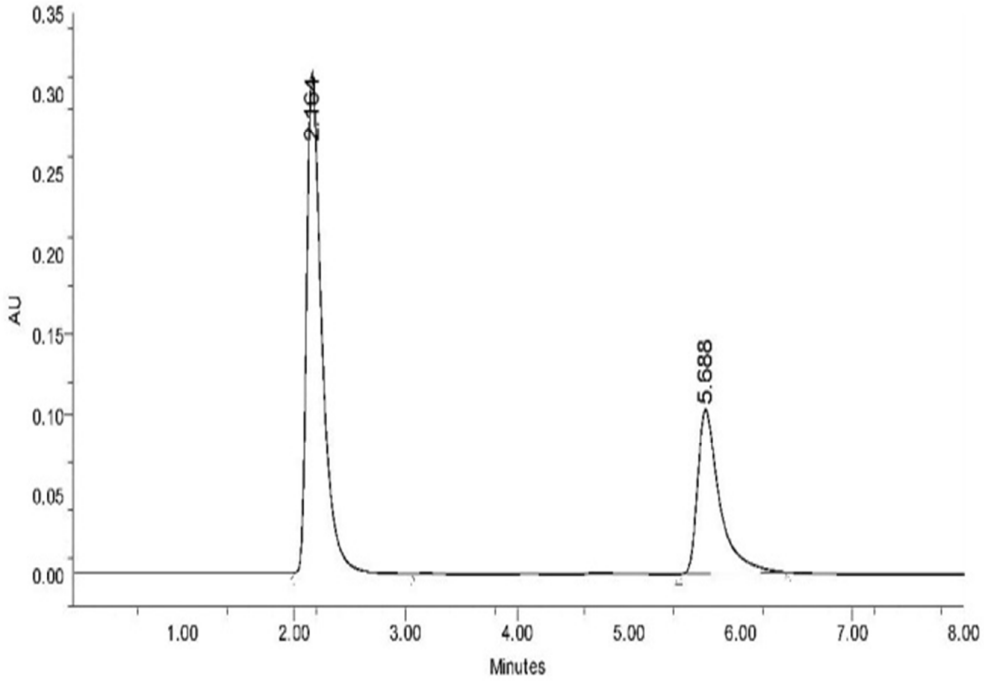
LINEARITY

Sample Name:	SIM SITA Linearty	Acquired By:	lab user
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	100, 101, 102, 103, 99	Acq. Method Set:	sita_simva
Injection #:	1, 2	Processing Method:	S_S LINEARITY
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

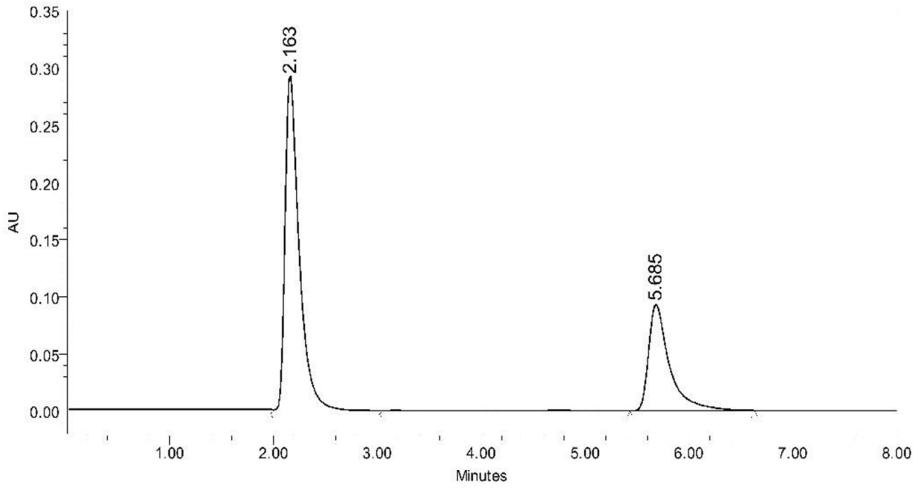


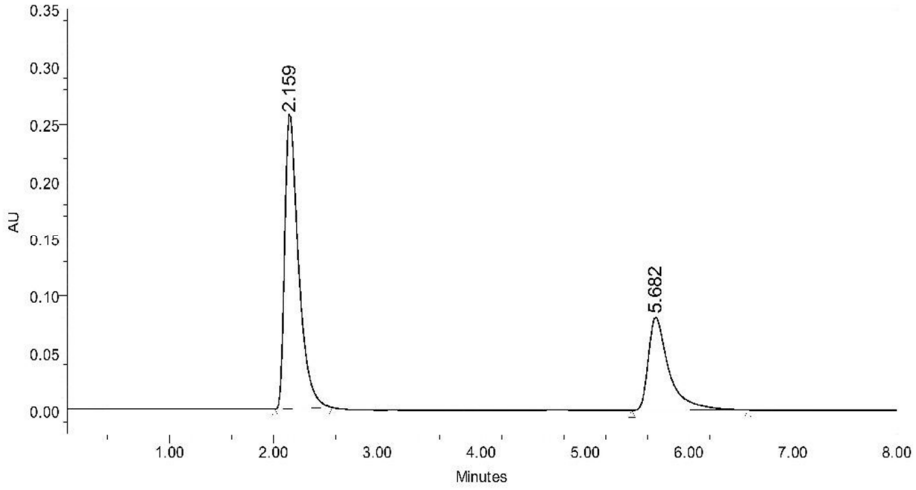
Chromatogram-23





Chromatogram-24&25





	Name	RT	Area	Height (µV)
1	Sitagliptin	2.158	2115842	226144
2	Simvastatin	5.690	1028433	80819
3	Sitagliptin	2.159	2360795	259812
4	Simvastatin	5.682	1117784	81097

	Name	RT	Area	Height (µV)
5	Sitagliptin	2.160	2567567	274081
6	Simvastatin	5.682	1205382	88542
7	Sitagliptin	2.163	2780462	292813
8	Simvastatin	5.685	1301561	93386

Chromatogram-26&27

	Name	RT	Area	Height (µV)
9	Sitagliptin	2.164	2986886	313584
10	Simvastatin	5.688	1403961	102979

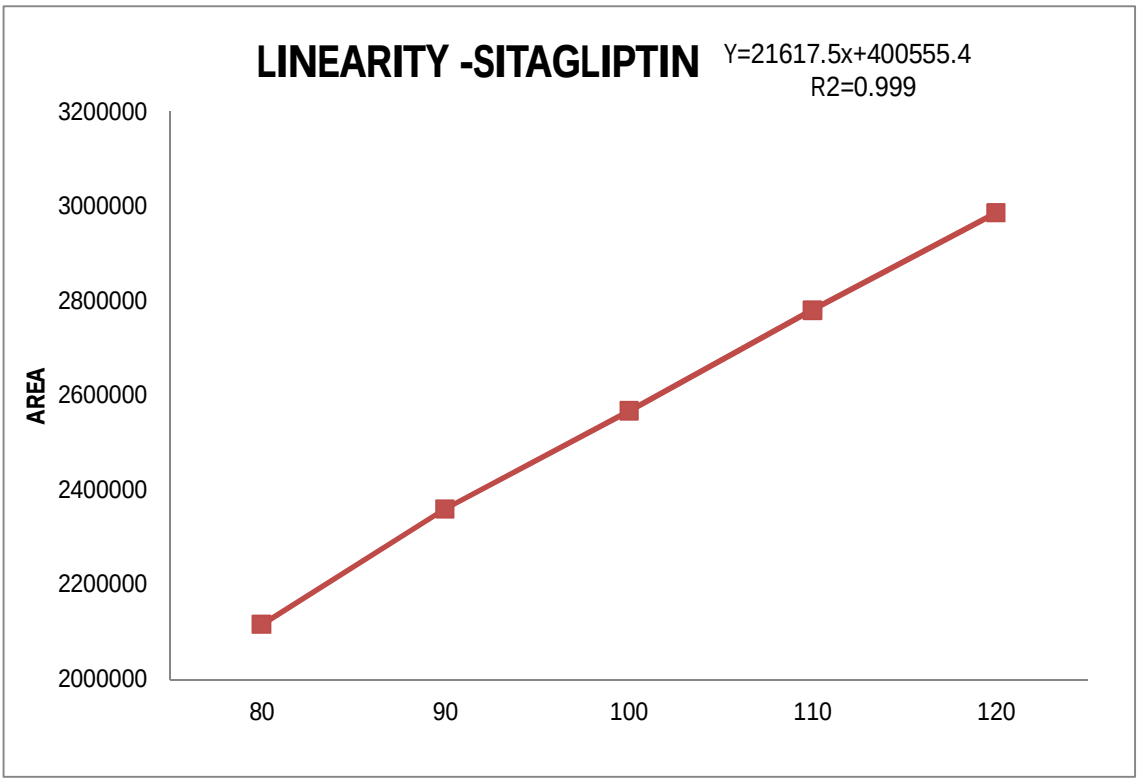
Linearity Results (for Sitagliptin)

Sr.no	Concentration	Area	Average area
1	80ppm	2115841	2115842
		2115843	
		2115844	
2	90ppm	2360794	2360795
		2360796	
		2360797	
3	100ppm	2567566	2567567
		2567568	
		2567569	
4	110ppm	2780461	2780462
		2780463	
		2780464	
5	120ppm	2986885	2986886
		2986887	
		2986888	
	Correlation coefficient		0.999

Table-4

Acceptance Criteria

- Correlation coefficient should be not less than 0.999.


LINEARITY -SITAGLIPTIN
Y=21617.5x+400555.4
R2=0.999
AREA
3200000
3000000
2800000
2600000
2400000
2200000
2000000
80
90
100
110
120

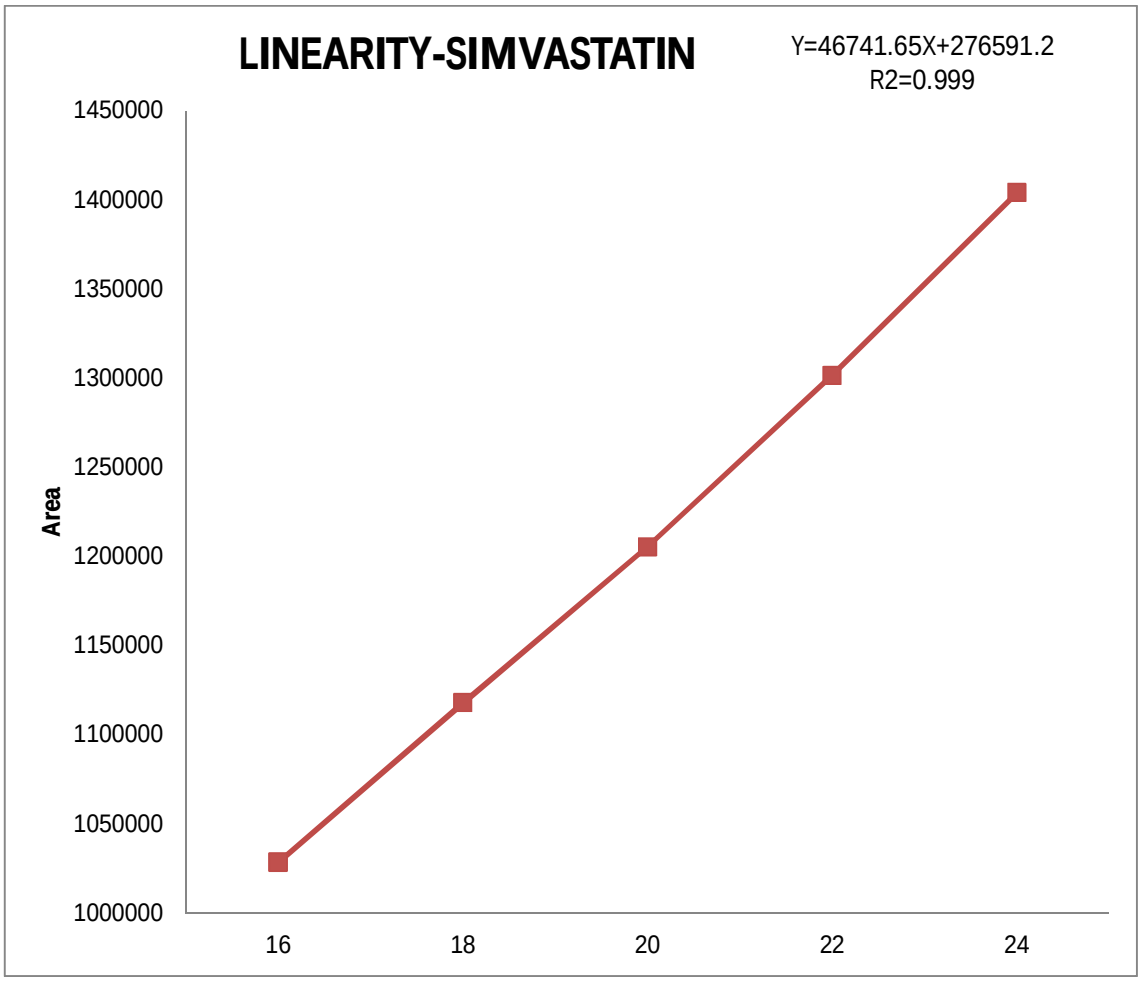
Linearity Results: (for Simvastatin)

S.No	Concentration	Area	Average area
1	16ppm	1028432	1028433
		1028434	
		1028435	
2	18ppm	1117783	1117784
		1117785	
		1117786	
3	20ppm	1205381	1205382
		1205383	
		1205384	
4	22ppm	1301560	1301561
		1301562	
		1301563	
5	24ppm	1403960	1403961
		1403962	
		1403963	
Correlation coefficient			0.999

Table-5

Acceptance Criteria

Correlation coefficient should be not less than 0.999.


LINEARITY-SIMVASTATIN
Y=46741.65X+276591.2
R2=0.999
Area
1450000
1400000
1350000
1300000
1250000
1200000
1150000
1100000
1050000
1000000
16
18
20
22
24

ASSAY

Standard drug solution

The standard stock solution was further diluted appropriately in mobile phase to get final concentration of 100mg/ml of Sitagliptin and 20mg/ml of Simvastatin.

Sample Stock solution

Twenty tablets were weighed and finely powdered. Accurately weighed and transferred a quantity of tablet powder equivalent to 100mg of Sitagliptin and 20mg of Simvastatin working sample into a 100ml clean dry volumetric flask containing mobile phase. The solution was sonicated for about 10mins and then made upto volume with mobile phase and filtered. Diluted 10ml of the above solution to 100ml with mobile phase.

Preparation of Sample solution

Diluted 5ml from the above solution to 10ml with the mobile phase in a 10ml volumetric flask.

Procedure

The chromatographic conditions were set as detailed under the study of linearity range and mobile phase was allowed to equilibrate with stationary phase. Equal volumes of standard and sample solution were injected separately. The chromatograms were recorded. The contents of Simvastatin and Sitagliptin were calculated

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weighed of working standard taken in mg.

P = Percentage purity of working standard.

LC = Label claim Sitagliptin mg/ml.

Assay Results (Sitagliptin)

$$\frac{2556801}{2596712} \times \frac{10}{10} \times \frac{1}{10} \times \frac{10}{56} \times \frac{10}{1} \times \frac{99.8}{100} \times 100 \times \frac{568}{100} = 99.67\%$$

Calculation (For Simvastatin)

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{Avg.\ Wt}{Label\ Claim} \times 100$$

Where

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weighed of working standard taken in mg.

P = Percentage purity of working standard

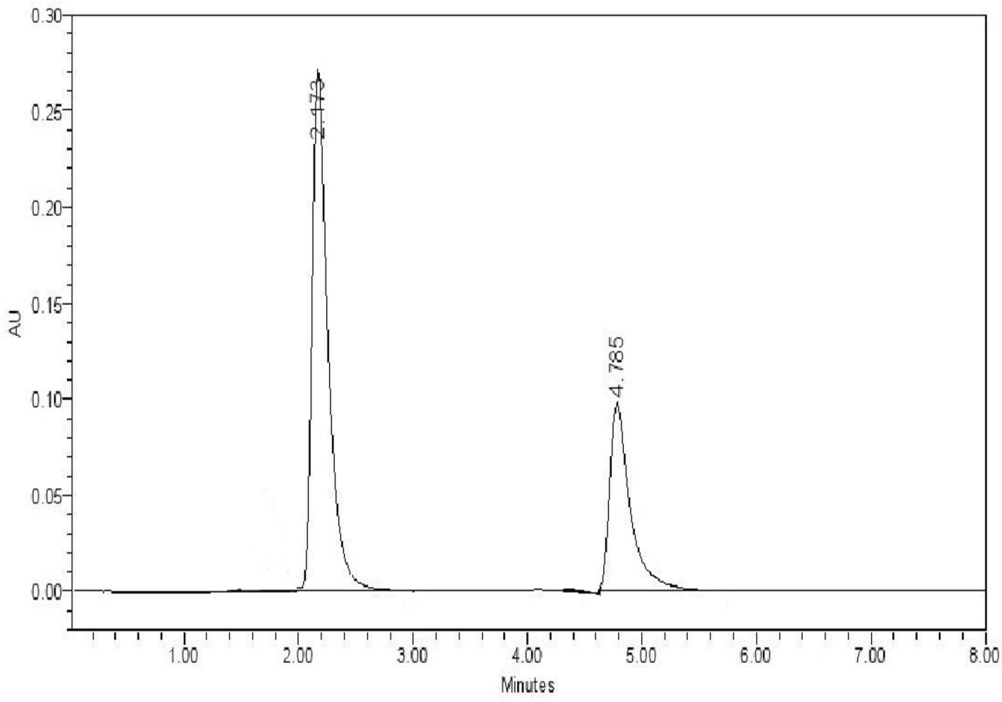
LC = Label claim of Simvastatin mg/ml.

Assay results

$$\frac{1234163}{1237668} \times \frac{10}{10} \times \frac{0.2}{10} \times \frac{10}{284} \times \frac{10}{0.2} \times \frac{99.7}{100} \times 100 \times \frac{568}{20} = 99.42\%$$

ASSAY-SAMPLE

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S Sample
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

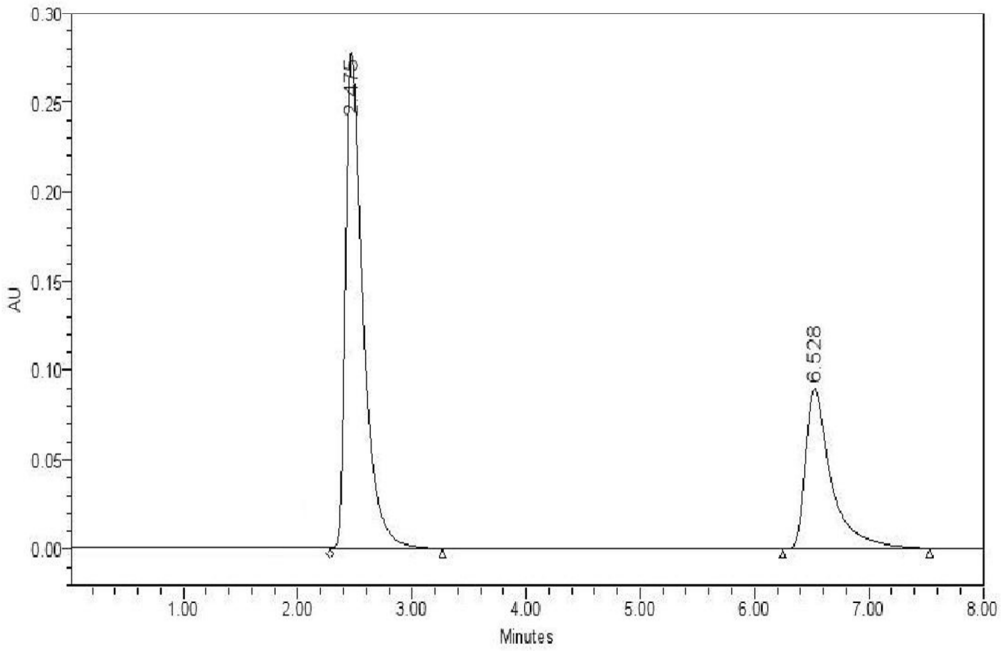


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.173	2556801	272458	1245.68		1.67
2	Simvastatin	4.785	1234163	98149	3986.32	9.06	1.92

Chromatogram-28

ASSAY STANDARD

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	10	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S Assay standard
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.475	2596712	280212	2326.64		1.12
2	Simvastatin	6.528	1237668	89515	4810.76	11.96	1.14

Chromatogram-29

Accuracy

Preparation of Standard stock solution

Accurately weighed and transferred 20 mg of Sitagliptin and Simvastatin working standard into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution.

Further pipetted 1.0ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation Sample solutions

For preparation of 50% solution (With respect to target Assay concentration)

Accurately weighed and transferred 10.0mg equivalent of Sitagliptin and Simvastatin into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock Solution)

Further pipetted 1.0ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration)

Accurately weighed and transferred 20mg of Sitagliptin and Simvastatin working standards into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Further pipetted 1.0ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration)

Accurately weighed and transferred 30mg of Sitagliptin and Simvastatin working standards into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Further pipetted 1.0ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

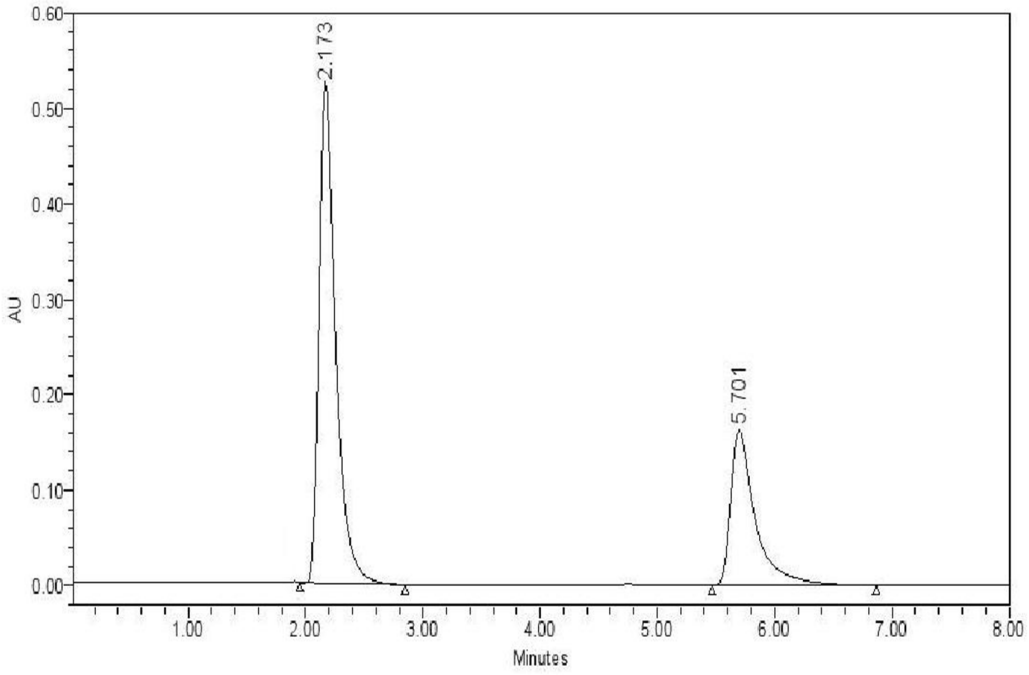
Procedure

Injected the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculated the Amount found and Amount added for Sitagliptin & Simvastatin and calculated the individual recovery and mean recovery values.

ACCURACY-STANDARD-1

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	104	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S ACC STD
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

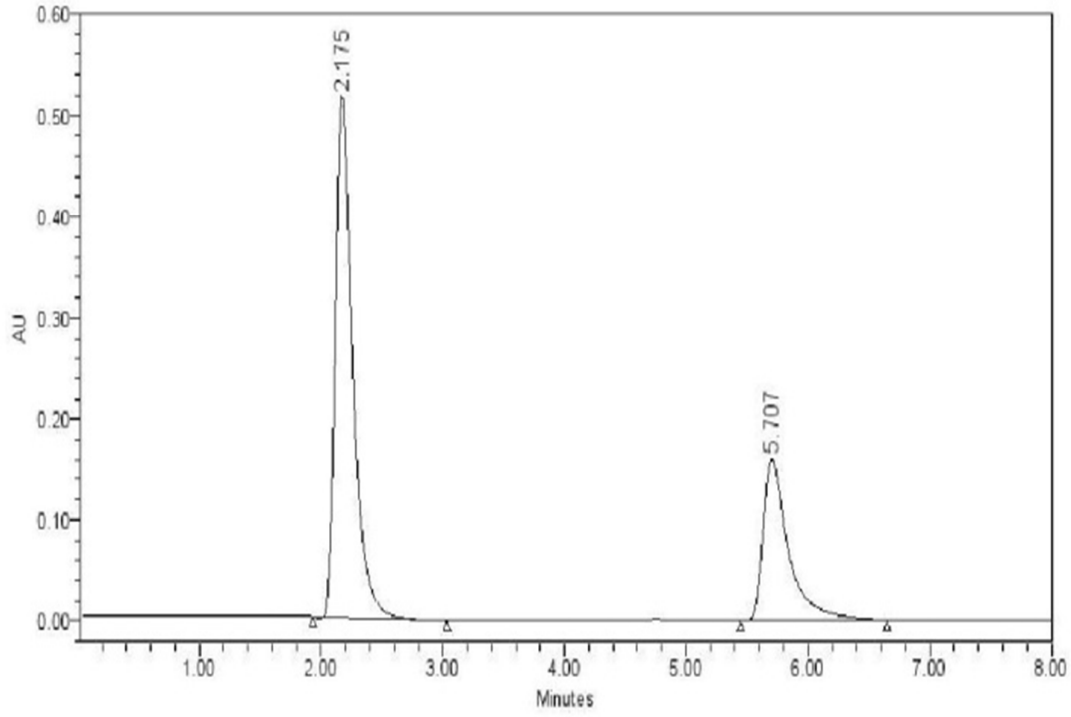


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.173	5172966	526318	2394.95		1.22
2	Simvastatin	5.701	2374610	162255	4301.94	11.27	1.06

Chromatogram-30

ACCURACY-STANDARD-2

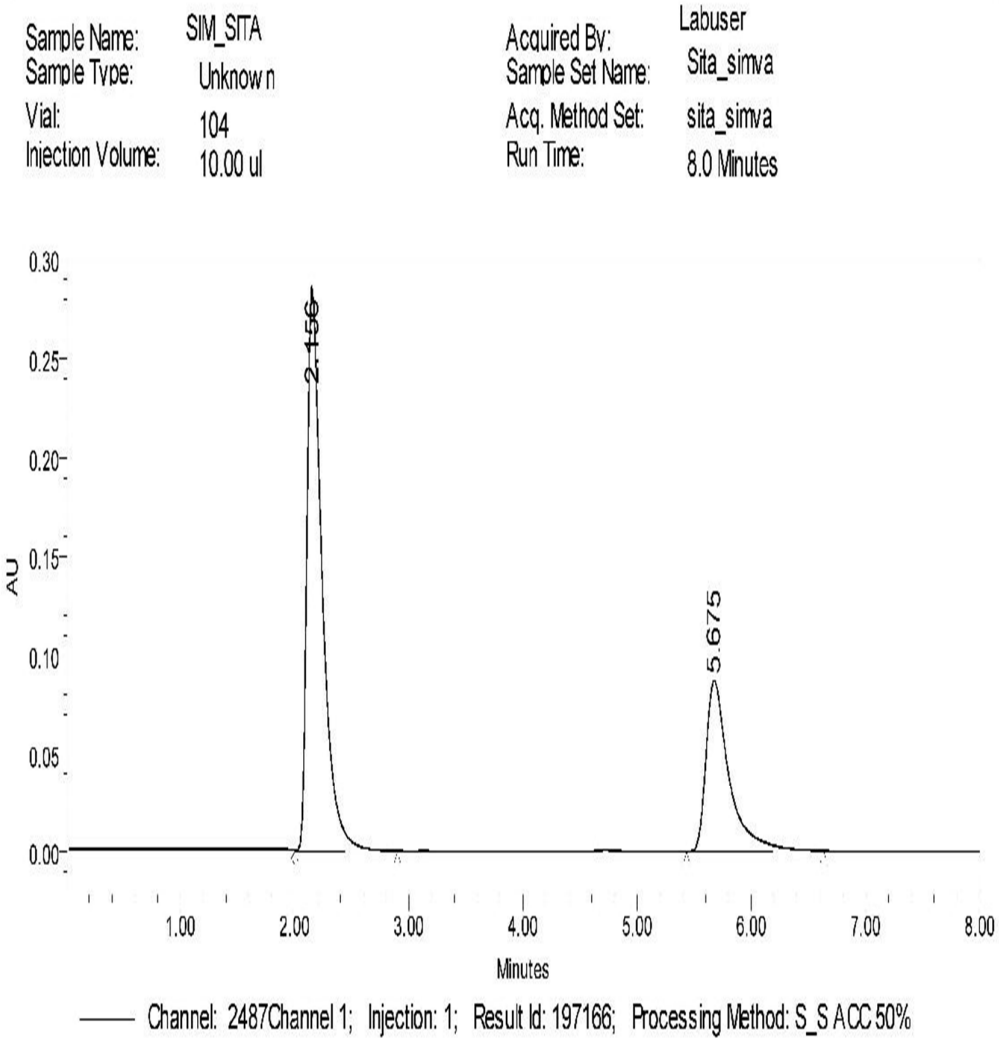
Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	104	Acq. Method Set:	sita_simva
Injection #:	2	Processing Method:	S_S ACC STD
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



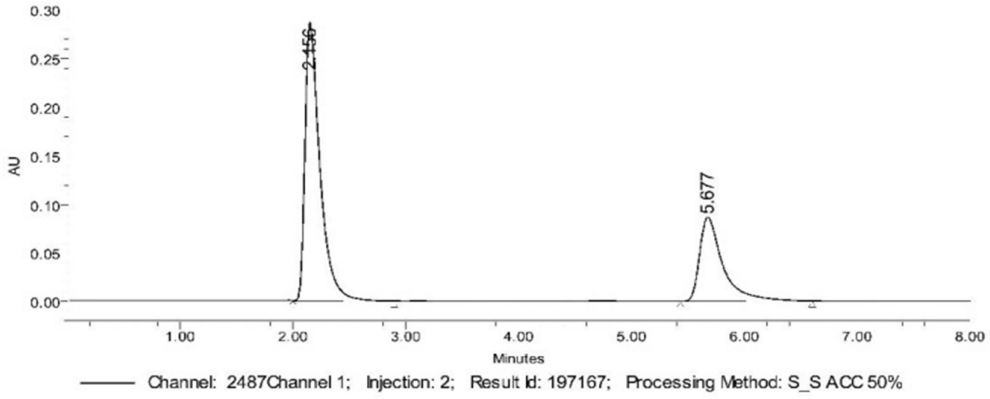
	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.175	5145179	520195	2394.95		1.22
2	Simvastatin	5.707	2346399	161069	4291.23	11.22	1.06

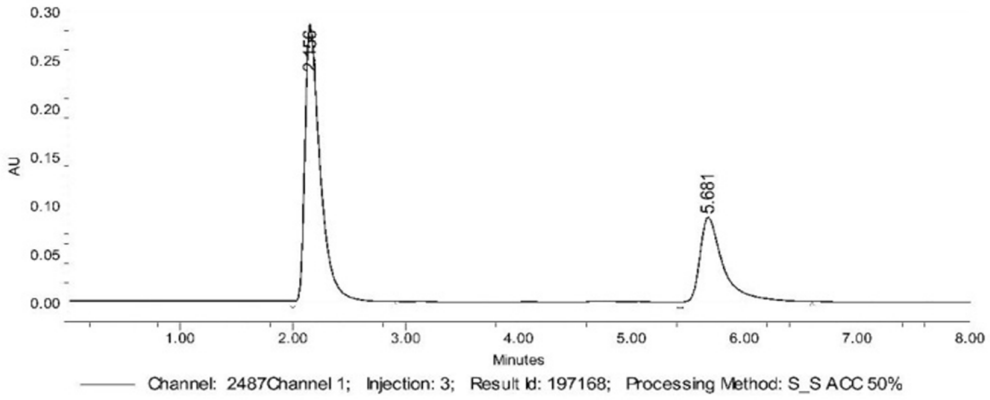
Chromatogram-31

ACCURACY-50%



Chromatogram-32





Name: Simvastatin

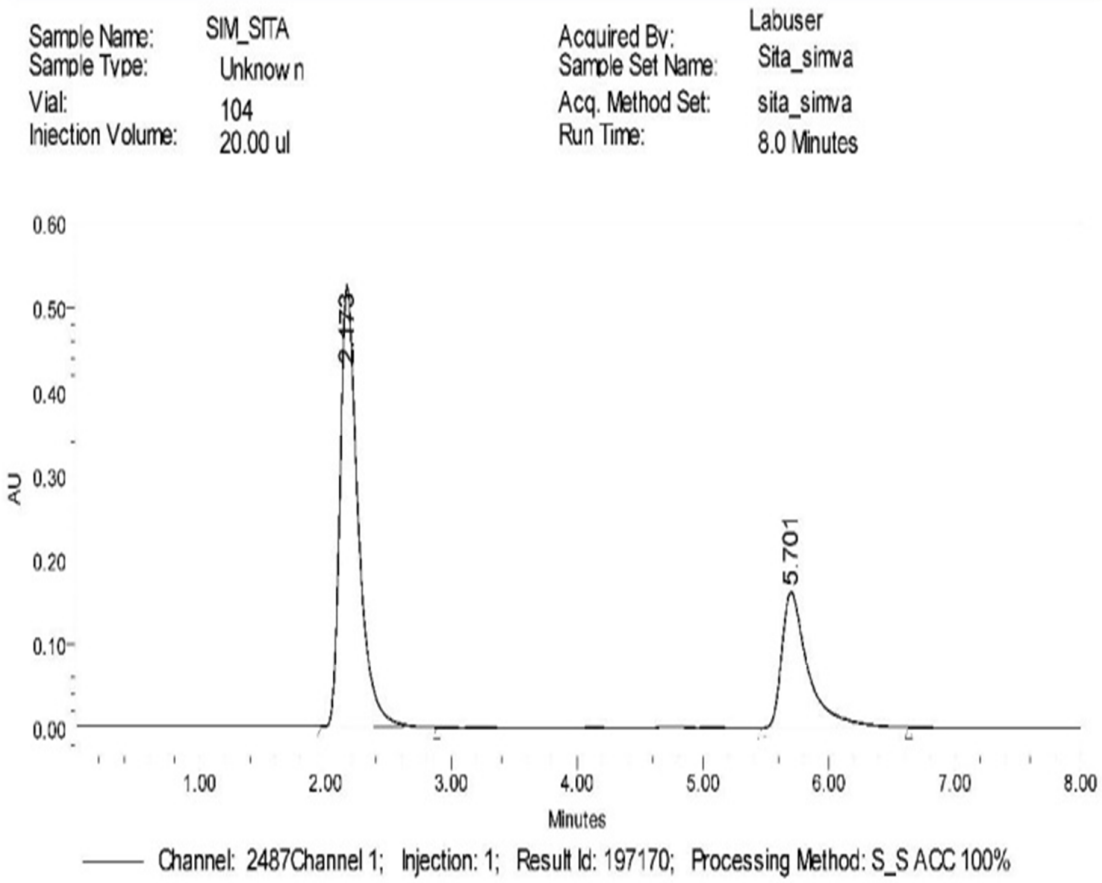
	Name	RT	Area	Height (µV)
1	Simvastatin	5.675	1239063	87381
2	Simvastatin	5.677	1238127	87526
3	Simvastatin	5.681	1241884	87164
Mean			1239691	
Std. Dev.			1955.7	
% RSD			0.16	

Chromatogram-33&34

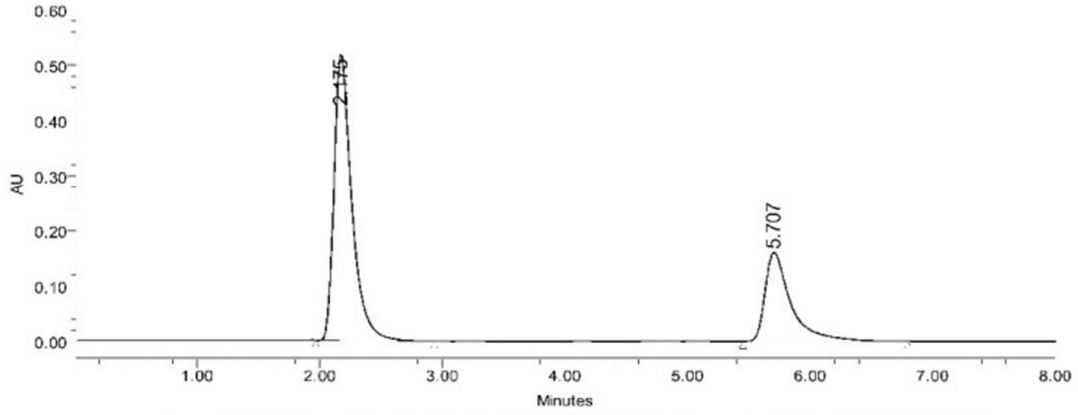
Name: Sitagliptin

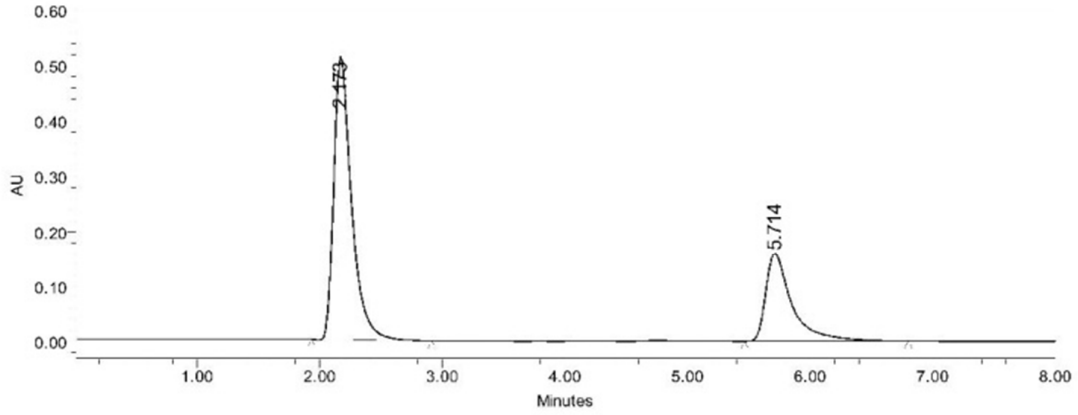
	Name	RT	Area	Height (µV)
1	Sitagliptin	2.156	2641004	287520
2	Sitagliptin	2.156	2630347	288086
3	Sitagliptin	2.156	2648612	287571
Mean			2639988	
Std. Dev.			9174.9	
% RSD			0.35	

ACCURACY-100%



Chromatogram-35





Name: Simvastatin

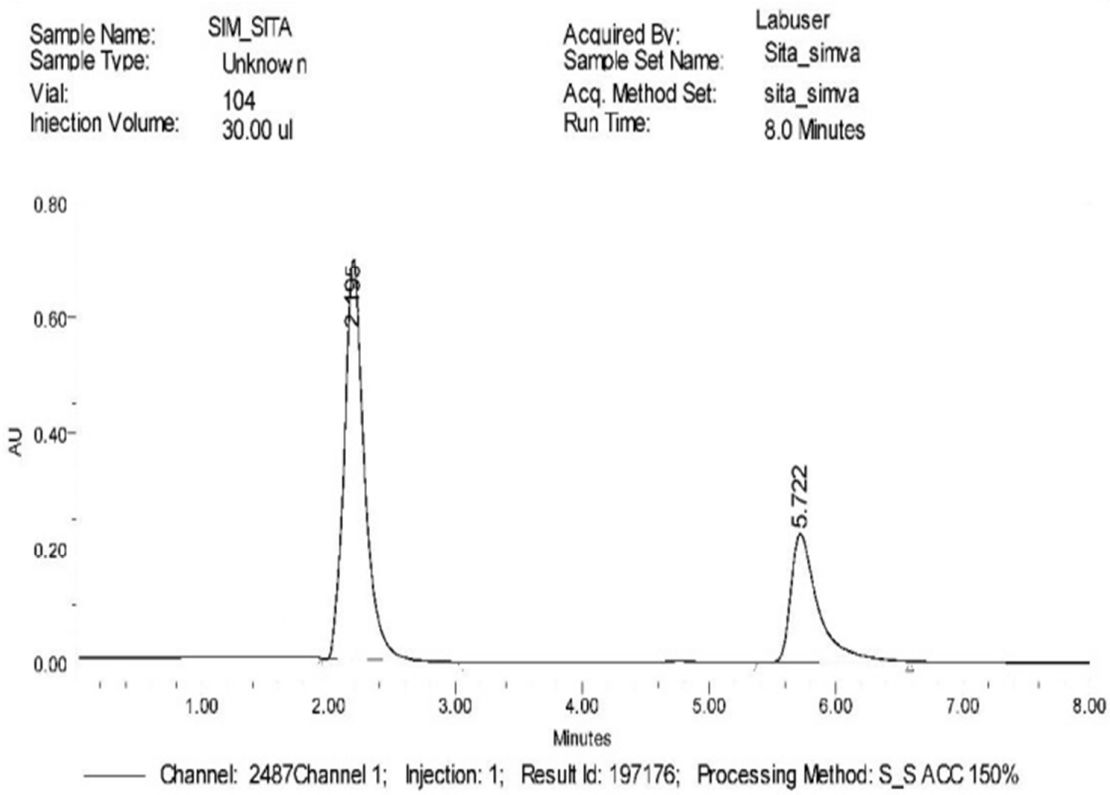
	Name	RT	Area	Height (µV)
1	Simvastatin	5.701	2350819	162139
2	Simvastatin	5.707	2364664	161167
3	Simvastatin	5.714	2362722	159738
Mean			2359402	
Std. Dev.			7496.1	
% RSD			0.32	

Chromatogram-36&37

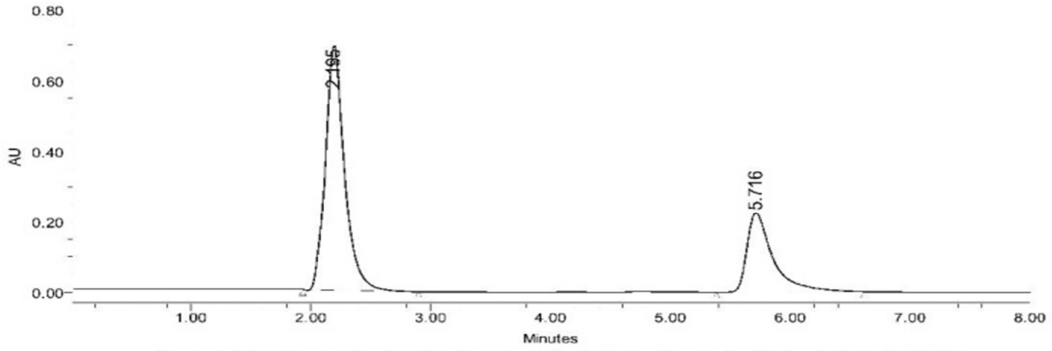
Name: Sitagliptin

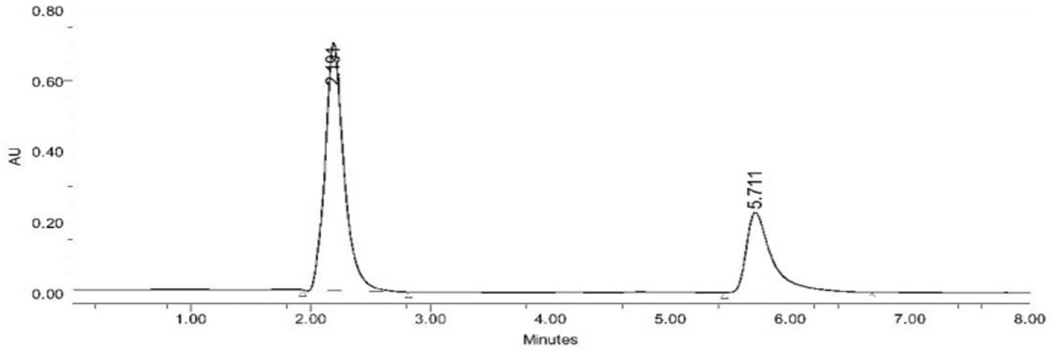
	Name	RT	Area	Height (µV)
1	Sitagliptin	2.173	5179472	526530
2	Sitagliptin	2.175	5161809	520799
3	Sitagliptin	2.173	5138918	514928
Mean			5160066	
Std. Dev.			20333.0	
% RSD			0.39	

ACCURACY-150%



Chromatogram-38





Name: Simvastatin

	Name	RT	Area	Height (µV)
1	Simvastatin	5.722	3333748	225335
2	Simvastatin	5.716	3353563	224437
3	Simvastatin	5.711	3385011	226601
Mean			3357441	
Std. Dev.			25850.5	
% RSD			0.77	

Chromatogram-39&40

Name: Sitagliptin

	Name	RT	Area	Height (µV)
1	Sitagliptin	2.195	7623823	697368
2	Sitagliptin	2.195	7656212	693248
3	Sitagliptin	2.191	7681778	704909
Mean			7653938	
Std. Dev.			29044.1	
% RSD			0.38	

The accuracy results for Sitagliptin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	2639988	10.0	10.21	102.14%	100.22%
100%	5160066	20.0	19.96	99.82%	
150%	7653938	30.0	29.61	98.71%	

Table-6

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102.0%.

The accuracy results for Simvastatin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1239691	10.4	10.47	100.69%	100.54%
100%	2359402	20.0	19.93	99.65%	
150%	3357441	28.0	28.36	101.29%	

Table-7

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102.0%**.**

System Precision

Preparation of stock solution

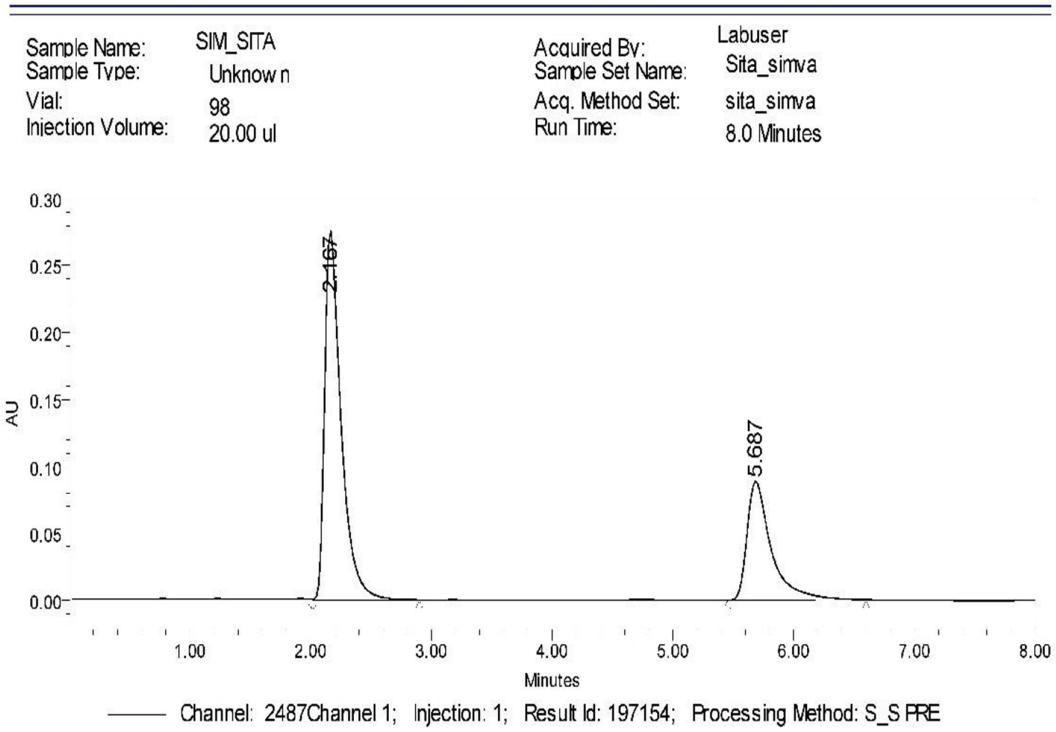
Accurately weighed and transferred 10 mg of Sitagliptin and Simvastatin working standard into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution).

Further pipetted 1ml of Sitagliptin & 0.2ml of Simvastatin of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

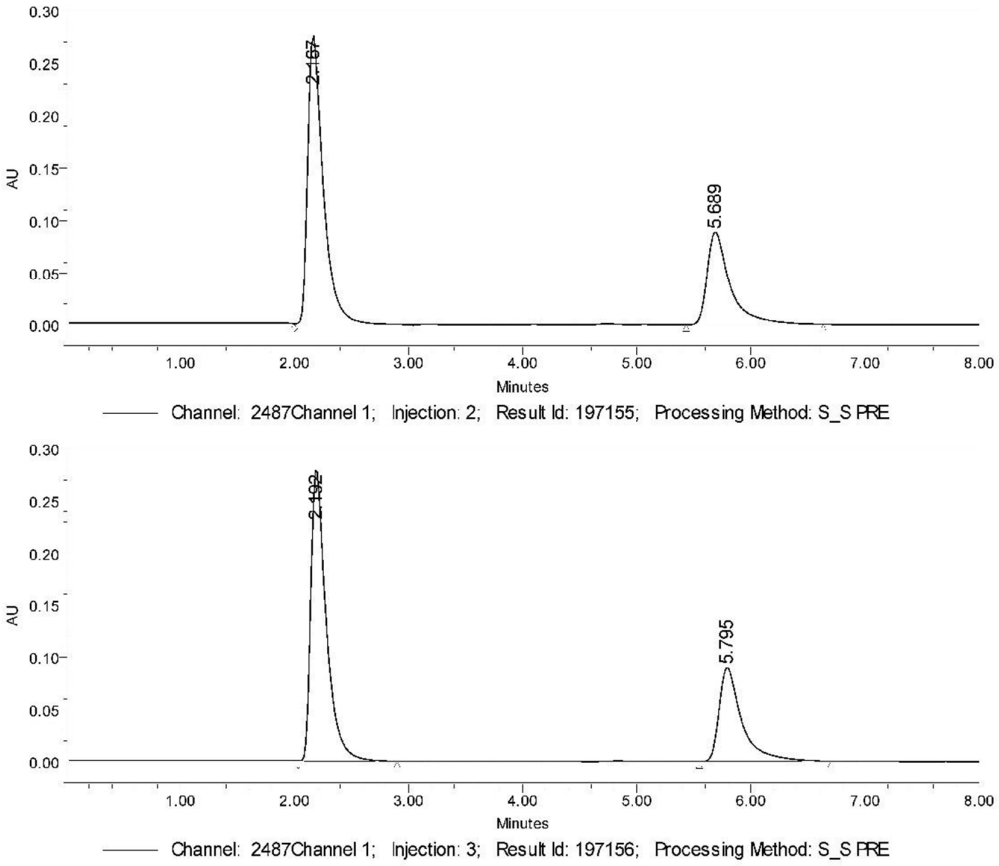
Procedure

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

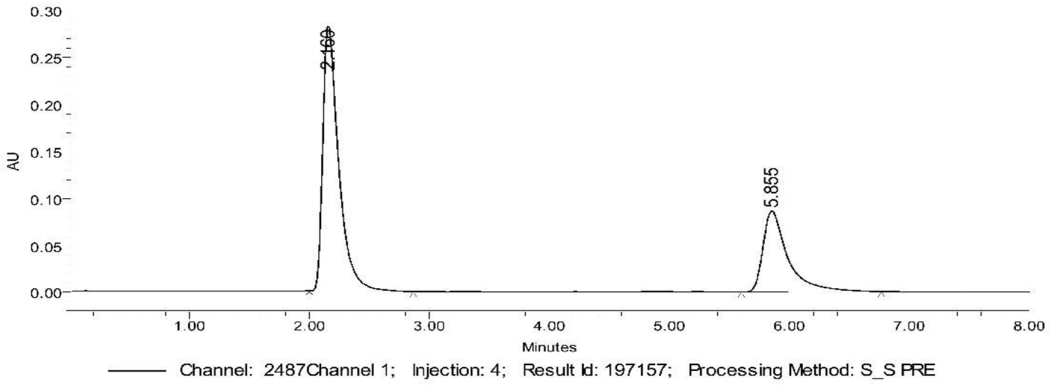
SYSTEM PRECISION

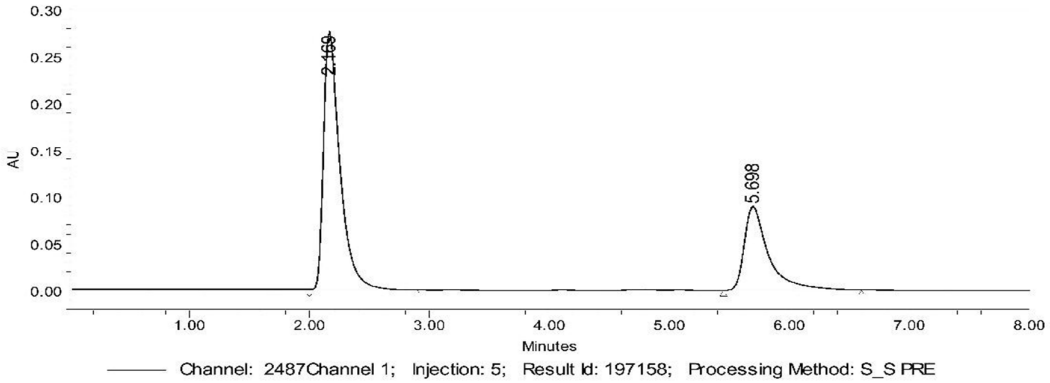


Chromatogram-41



Chromatogram-42&43





Name: Sitagliptin

	Name	RT	Area	Height (µV)
1	Sitagliptin	2.167	2600572	275579
2	Sitagliptin	2.167	2624671	275369
3	Sitagliptin	2.192	2617396	282564
4	Sitagliptin	2.160	2610977	287122
5	Sitagliptin	2.169	2619579	276984
Mean			2614639	
Std. Dev.			9272.7	

	Name	RT	Area	Height (µV)
% RSD			0.35	

Chromatogram-44&45

Name: simvastatin

	Name	RT	Area	Height (µV)
1	simvastatin	5.687	1240270	89192
2	simvastatin	5.689	1245251	89257
3	simvastatin	5.795	1247103	89748
4	simvastatin	5.855	1245022	87264
5	simvastatin	5.698	1245921	89113
Mean			1244713	
Std. Dev.			2612.3	
% RSD			0.21	

The results are summarized for Sitagliptin and Simvastatin

Sr.no	Sitagliptin	Simvastatin
1	2600572	1240270
2	2624671	1245251
3	2617396	1247103
4	2610977	1245022
5	2619579	1245921
Average	2614639	1244713
Standard Deviation	9272.7	2612.3
%RSD	0.35	0.21

Table-8

Acceptance Criteria

The % RSD for the area of five standard injections results should not be more than 2%.

Method Precision

Six sample preparations of the Simvastatin and Sitagliptin tablets shall be prepared and analyzed as per the method.

Preparation of Sample Stock Solution

Accurately weighed and transferred the powder of sample equivalent to 100mgof Sitagliptin and 20mg separately six times into a different 50ml clean dry volumetric flask and added mobile phase. The solutions was sonicated for about 10mins and then made upto volume with mobile phase. Diluted 5ml of the above solution to 50ml with mobile phase.

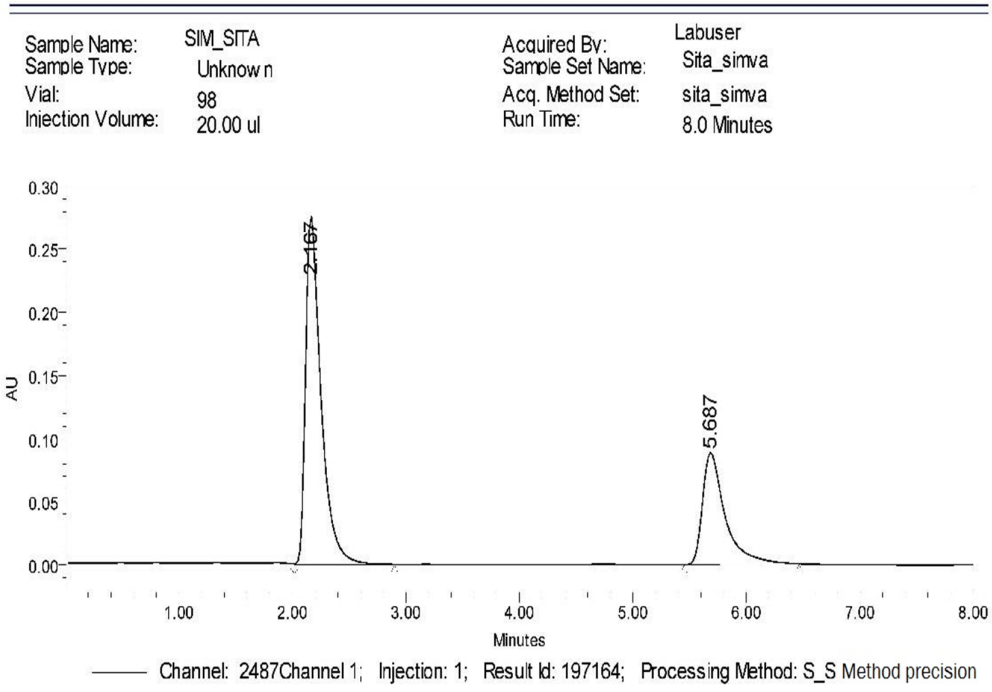
Preparation of sample solution

Diluted 5ml from the above solutions to 10ml with the mobile phase in a 10ml volumetric flask.(100mgSitagliptin&20 mg of Simvastatin).

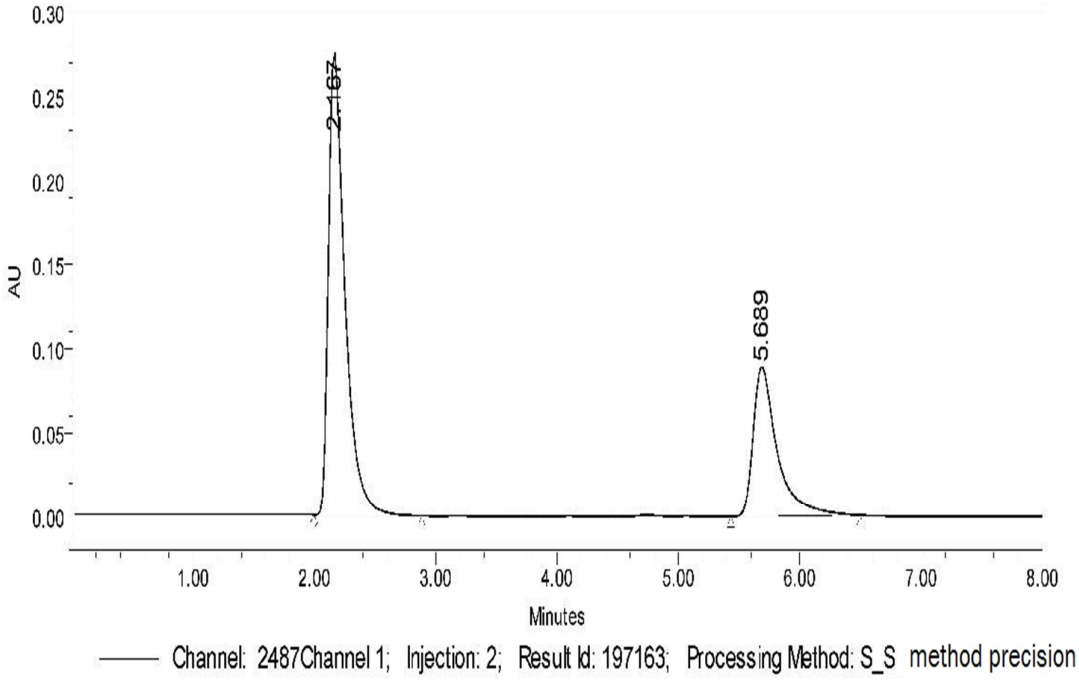
Procedure

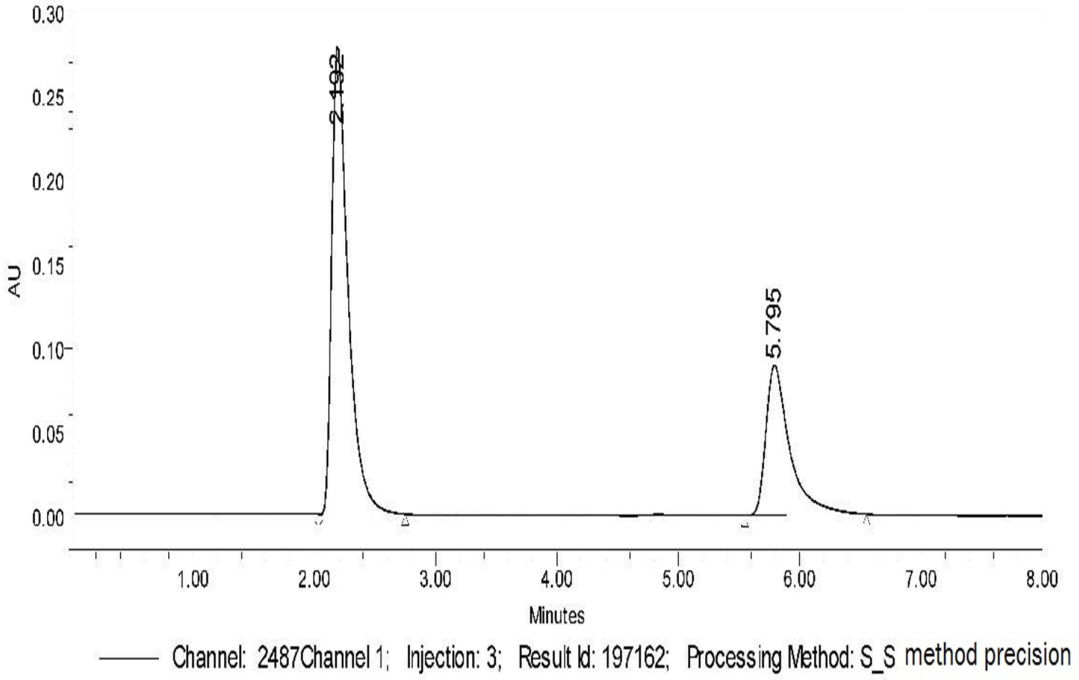
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

METHOD PRECISION

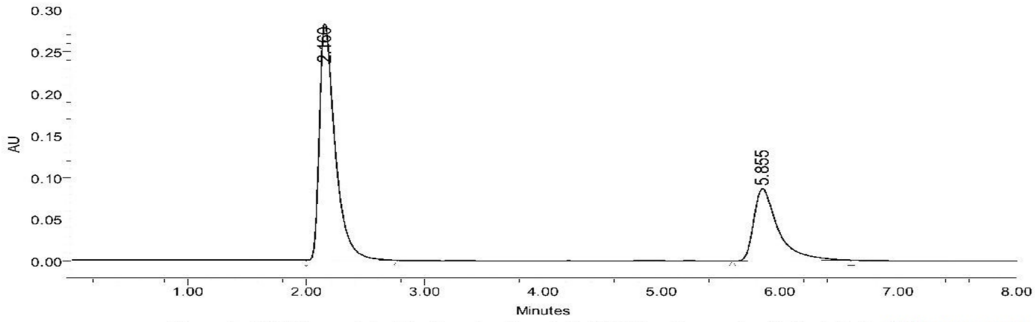


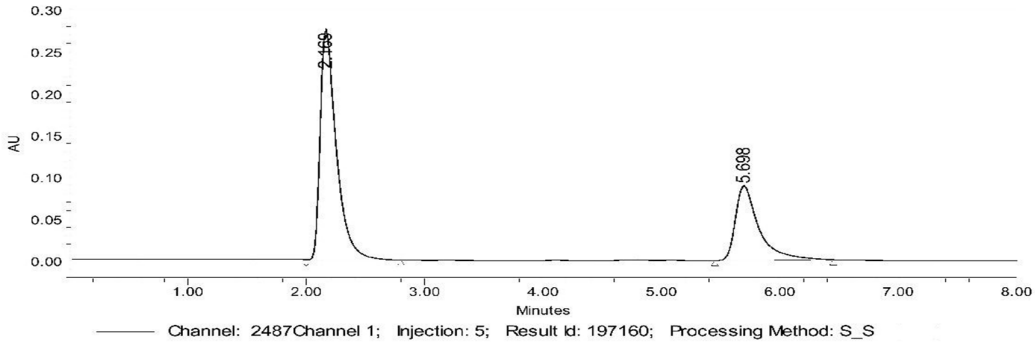
Chromatogram-46





Chromatogram-47&48





Name: Simvastatin

	Name	RT	Area	Height (µV)
1	Simvastatin	5.687	1225584	89092
2	Simvastatin	5.689	1231724	89166
3	Simvastatin	5.795	1228089	89611
4	Simvastatin	5.855	1223118	87100
5	Simvastatin	5.698	1226449	88971
Mean			1226993	
Std. Dev.			3196.9	

	Name	RT	Area	Height (µV)
% RSD			0.26	

Chromatogram-49&50

Name: Sitagliptin

	Name	RT	Area	Height (µV)
1	Sitagliptin	2.167	2600572	275579
2	Sitagliptin	2.167	2607709	275175
3	Sitagliptin	2.192	2599166	282270
4	Sitagliptin	2.160	2601972	286981
5	Sitagliptin	2.169	2609884	276842
Mean			2603861	
Std. Dev.			4677.3	
% RSD			0.18	

The results are summarized for Sitagliptin and Simvastatin

Sr.no	Sitagliptin Area	Simvastatin area
1	2600572	1225584
2	2607709	1231724
3	2599166	1228089
4	2601972	1223118
5	2609884	1226449
Average	2603861	1226993
Standard Deviation	4677.3	3196.9
%RSD	0.18	0.26

Table-9

Acceptance Criteria

The % RSD for the area of five standard injections results should not be more than 2%.

Sensitivity

The Sensitivity of measurement of Simvastatin and Sitagliptin by use of the proposed method was estimated in terms of the limit of detection (LOD) and the Limit of Quantitation (LOQ). The LOD and LOQ were calculated by the use of the equations LOD = 3.3 × σ / S and LOQ = 10 × σ / S where σ is the standard deviation of intercept of Calibration plot and S is the average of the slope of the corresponding Calibration plot.

Limit of quantification (For Sitagliptin)

Preparation of 100µg/ml solution

Accurately weighed and transferred 10mg of Sitagliptin sample into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Further pipetted 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 0.4% solution At Specification level (0.4µg/ml solution)

Further pipetted 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Pipetted 0.4mL of 10µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

Limit of quantification (For Simvastatin)

Preparation of 20µg/ml solution

Accurately weighed and transferred 10mg of Simvastatin sample into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Further pipetted 0.2ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

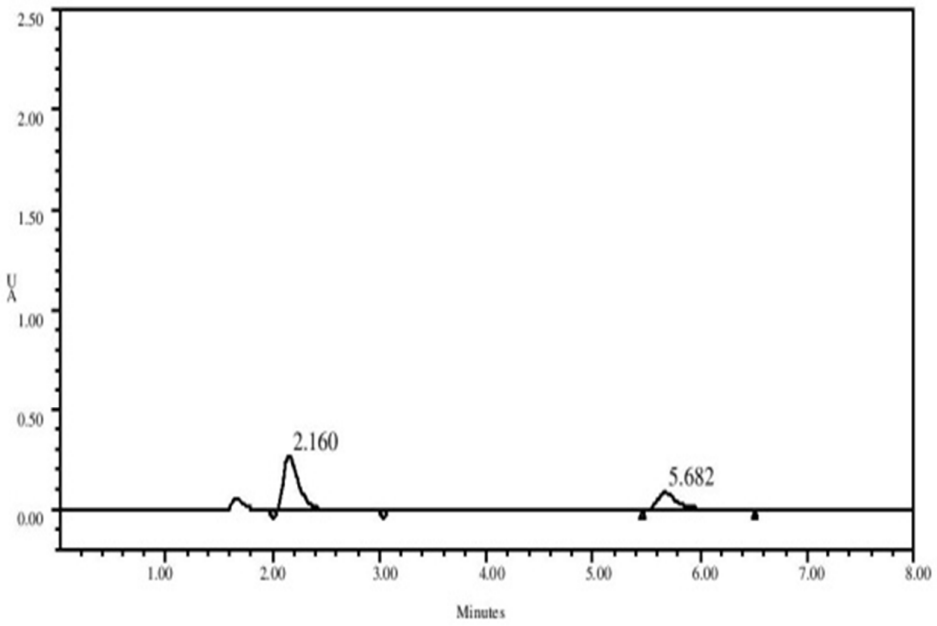
Preparation of 1.2% solution at Specification level (0.24µg/ml solution)

Further pipetted 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Pipetted 1.2mL of 2µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

LIMIT OF QUANTIZATION

Sample Name:	SIM_SITA	Acquired By:	lab user
Sample Type:	Unknown	Sample Set Name:	Sim_sita
Vial:	101	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S LOQ
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



	Name	RT	Area	Height (V)
1	Sitagliptin	2.160	8799	945
2	Simvastatin	5.682	12219	951

Chromatogram-51

Calculation of S/N Ratio for Sitagliptin

Average Baseline Noise obtained from Blank : 95 µV

Signal Obtained from LOQ solution (0.4% of target assay concentration) : 945µV

S/N = 945/95 = 9.95.

Acceptance Criteria

S/N Ratio value shall be 10 for LOQ solution.

Calculation of S/N Ratio for Simvastatin

Average Baseline Noise obtained from Blank : 95 µV

Signal Obtained from LOQ solution (1.2% of target assay concentration) : 951µV

S/N = 951/95 = 10.01.

Acceptance Criteria

S/N Ratio value shall be 10 for LOQ solution.

Limit of detection (for Sitagliptin)

Preparation of 100µg/ml solution

Accurately weighed and transferred 10mg of Sitagliptin working standard into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Further pipetted 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 0.13% solution At Specification level (0.13µg/ml solution)

Further pipetted 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Pipetted 0.13mL of 10µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

Limit of detection (for Simvastatin)

Preparation of 20µg/ml solution

Accurately weighed and transferred 10mg of Simvastatin Sample into a 10mL clean dry volumetric flask added about 7mL of Diluent and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution).

Further pipetted 0.2ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

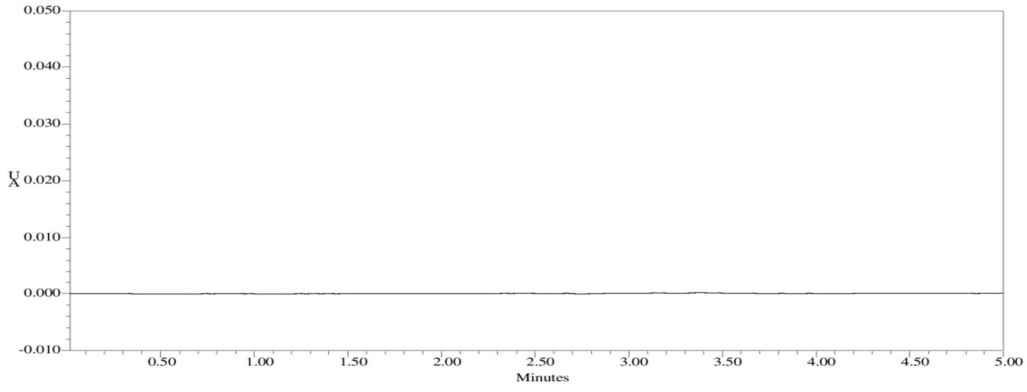
Preparation of 0.35% solution At Specification level (0.07µg/ml solution)

Further pipetted 1ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent.

Pipetted 0.35mL of 2µg/ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent.

BLANK FOR LOD & LOQ

Sample Name:	blk	Acquired By :	System
Sample Type:	Unknown	Sample Set Name:	
Vial:	54	Acq. Method Set :	Sit-Sim method
Injection #:	1	Processing Method:	Sit-Sim
Injection Volume:	20.00 ul	Channel Name :	2487Channel 1
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	254nm

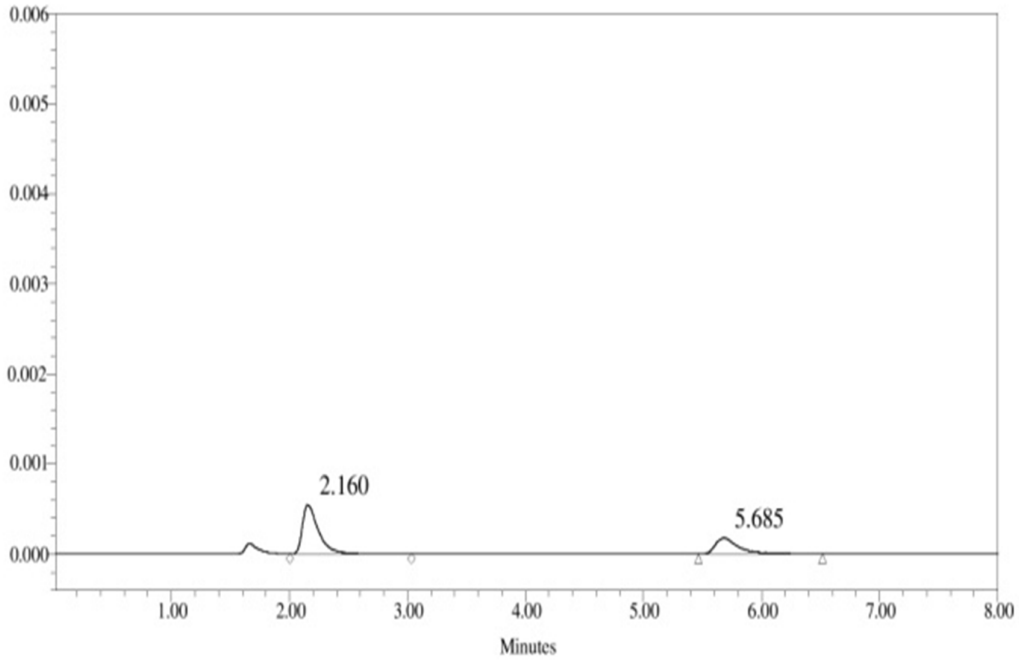


	Baseline Noise (mV)
1	0.095

Chromatogram-52

LIMIT OF DETCTION

Sample Name:	SIM_SITA	Acquired By:	lab user
Sample Type:	Unknown	Sample Set Name:	Sim_sita
Vial:	101	Acq. Method Set:	sita_simva
Injection #:	1	Processing Method:	S_S LOD
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



	Name	RT	Area	Height (V)
1	Sitagliptin	2.160	2653	285
2	Simvastatin	5.682	3828	298

Chromatogram-53

Calculation of S/N ratio for Sitagliptin

Average Baseline Noise obtained from Blank : 95 µV

Signal Obtained from LOD solution (0.13% of target assay concentration) : 285 µV

S/N = 285/95 = 3.00

Acceptance Criteria

S/N Ratio value shall be 3 for LOD solution.

Calculation of S/N ratio Simvastatin

Average Baseline Noise obtained from Blank : 95 µV

Signal Obtained from LOD solution (0.35% of target assay concentration) : 298 µV

S/N = 298/95 = 3.14

Acceptance criteria

S/N Ratio value shall be 3 for LOD solution.

Robustness

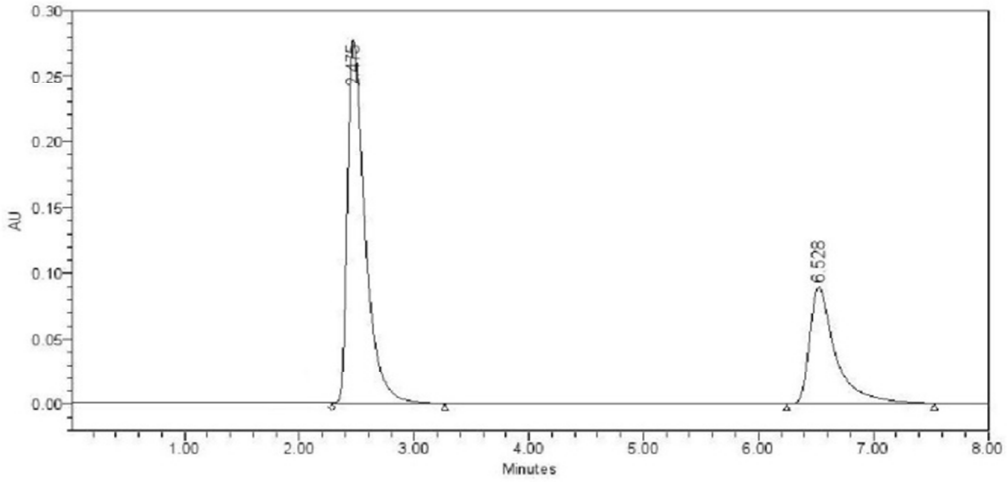
As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

a. The flow rate was varied at 0.7 ml/min to 0.9ml/min.

Standard solution 100ppm of Sitagliptin & 20ppm of Simvastatin was prepared and analysed using the varied flow rates along with method flow rate.

ROBUSTNESS

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simva less flow
Injection #:	1	Processing Method:	S_S LESS FLOW
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

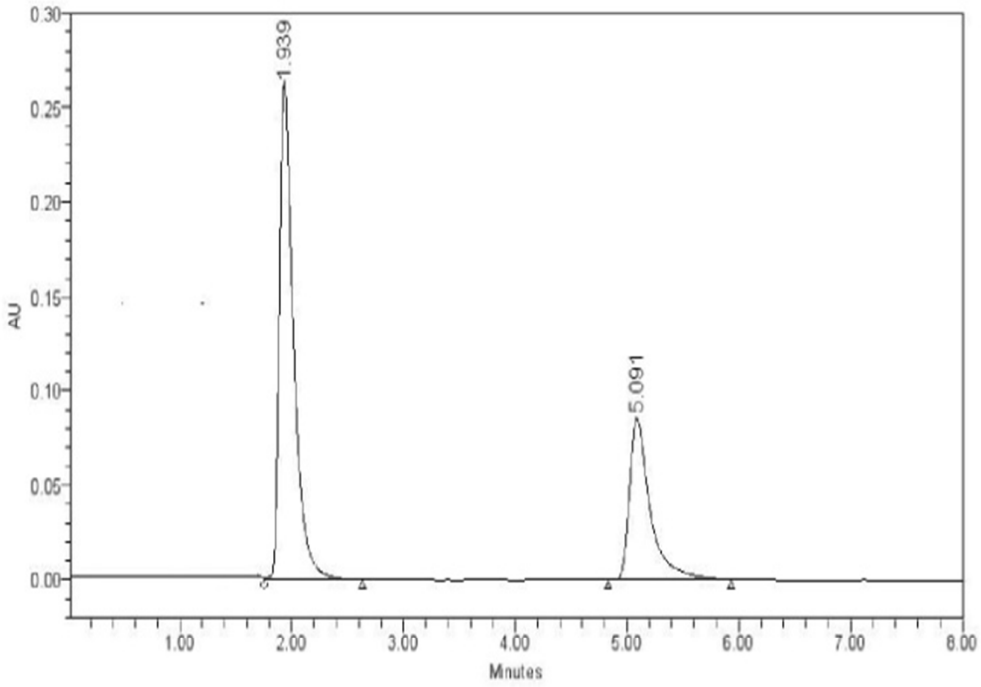


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.475	2975037	280212	2326.67		1.13
2	Simvastatin	6.528	1417227	89515	4810.76	11.99	1.19

Chromatogram-54

ROBUSTNESS

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simva more flow
Injection #:	1	Processing Method:	S_SMOREFLOW
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	1.939	2323619	265234	2204.28		1.14
2	Simvastatin	5.091	1104266	85708	4403.29	11.34	1.13

Chromatogram-55

The results are summarized

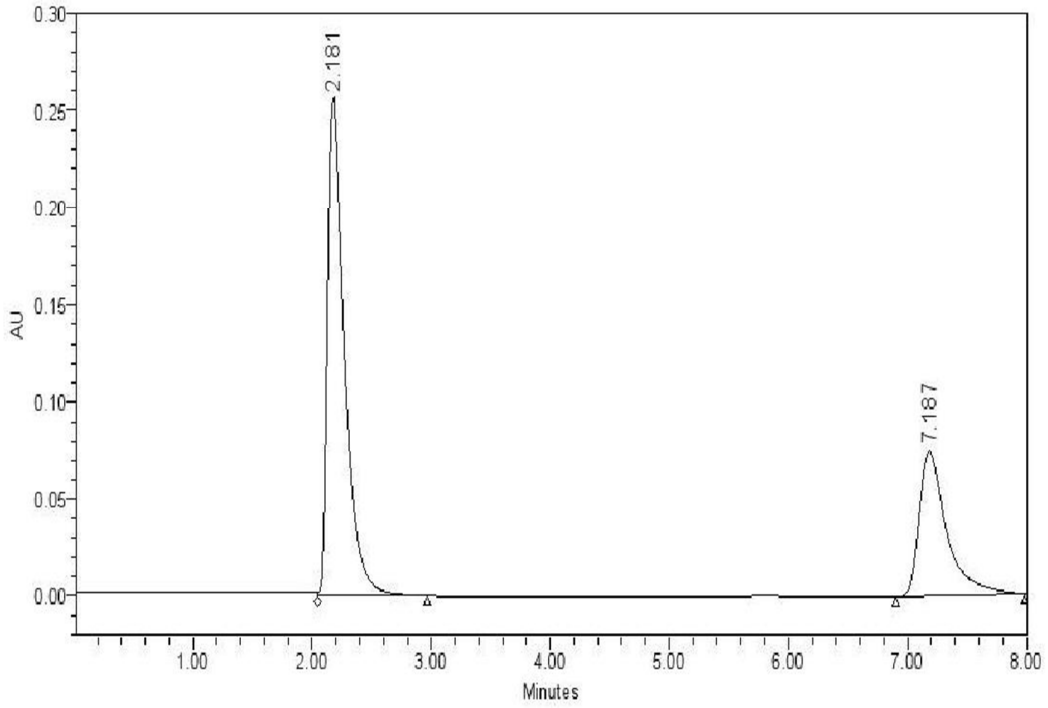
On evaluation of the above results, it can be concluded that the variation in flow rate does not affect the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±0.1%.

b. The Organic composition in the Mobile phase was varied from 70% to 80%.

standard solution 100 µg/ml of Sitagliptin & 20ppm of Simvastatin was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

ROBUSTNESS

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simva less org
Injection #:	1	Processing Method:	S_S LESS ORG
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254

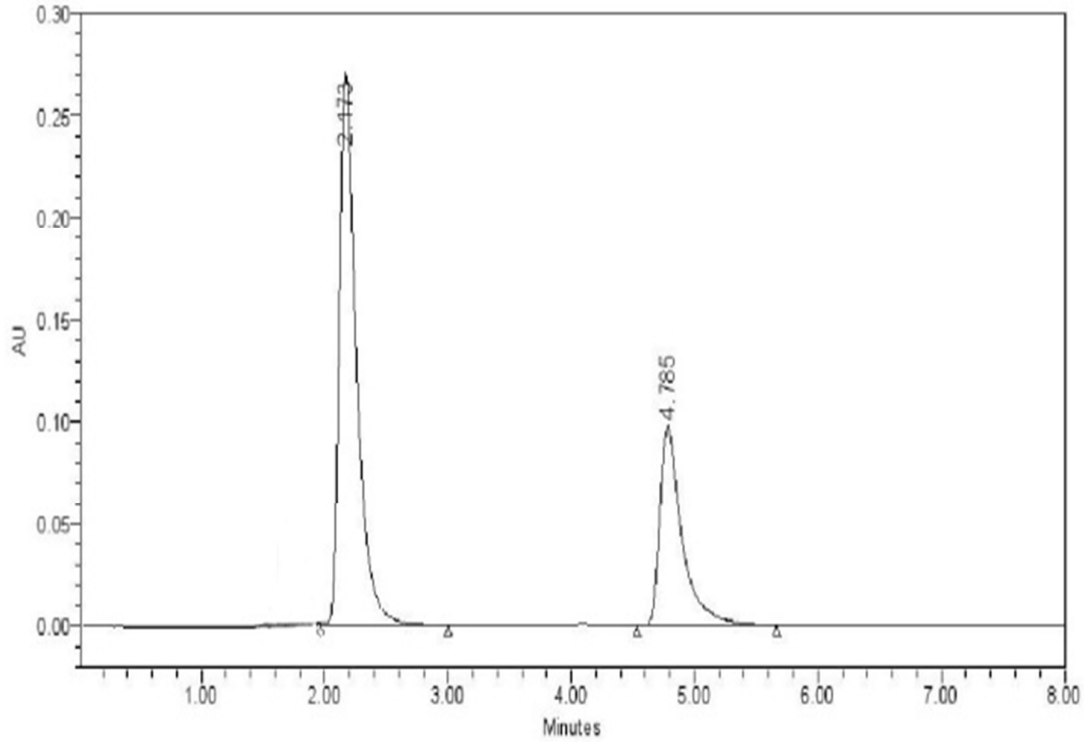


	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.181	2563842	256832	2149.58		1.10
2	Simvastatin	7.187	1210154	75167	5481.20	14.89	1.11

Chromatogram-56

ROBUSTNESS

Sample Name:	SIM_SITA	Acquired By:	Labuser
Sample Type:	Unknown	Sample Set Name:	Sita_simva Sample set
Vial:	105	Acq. Method Set:	sita_simva more org
Injection #:	1	Processing Method:	S_S MORE ORG
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.0 Minutes	Proc. Chnl. Descr.:	254



	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Plate Count	USP Resolution	USP Tailing
1	Sitagliptin	2.173	2644123	272443	1245.53		1.68
2	Simvastatin	4.785	1229838	98152	3986.48	9.05	1.93

Chromatogram-57

The results are summarized

On evaluation of the above results, it can be concluded that the variation in 5% Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±5%

Robustness results for Sitagliptin

Serial number	Parameter variation		Area of Sitagliptin	% RSD
1.	Flow rate	0.7	2975037	0.1165
			2975108	
			2975087	
			232619	0.1281
		0.9	232605	
			232622	
2.	Organic phase	70	2563842	0.1185
			2563951	
			2563901	
		80	2644123	0.1321
			2645456	
			2643849	

Table-10

Results for actual Mobile phase composition (75:25 Acetonitrile: Buffer) have been considered from Assay standard.

Robustness results for Simvastatin

Serial number	Parameter variation		Area of Sitagliptin	% RSD
1.	Flow rate	0.7	1417227	0.124
			1417442	
			1417589	
		0.9	1104266	0.269
			1104236	
			1104671	
2.	Organic phase	70	1210154	0.198
			1211123	
			1242810	
		80	1229838	0.312
			1230152	
			1231268	

Table-11

Results for actual Mobile phase composition (75:25 Acetonitrile: Buffer) have been considered from Assay standard.

Chapter-6

Results & discussion

Results and discussion

6.1. Results Of Validation

The following validation parameters were performed and the results obtained were observed to be in the given ICH limits.

Sr.no	parameter	Acceptance Limit	Observed values	
			Sitagliptin	Simvastatin
1.	System suitability	%RSD should be NMT 2	%RSD 1.785979443	%RSD 1.062447522
2.	Linearity	Correlation coefficient should be not less than 0.999	0.999	0.999
3.	Assay	%Purity-(97%-103%)	99.67%	99.42%
4.	Accuracy	%Recovery range is 90-110%	100.22	100.54
5.	System precision	%RSD should be NMT 2	0.35	0.21
6.	Method precision	%RSD should be NMT 2	0.16	0.25

7.	LOD	S/N ratio shall be 3	3	3.14

8.	LOQ	S/N ratio Shall be 10	9.05	10.01

			Flow rate		Wavelength	
			Less flow	More flow	Less organic	More organic
9.	Robustness	% RSD shall not be more than 5	0.7-0.1165	0.9-0.1281	241-0.1185	267-0.1321
			0.7-0.124	0.9-0.269	241-0.198	267-0.312

Table -12

For routine analytical purpose, it is always necessary to establish methods capable of analyzing huge number of samples in a short time period with due accuracy and precision.

Sitagliptin and Simvastatin are official in Indian Pharmacopoeia. There is one spectroscopic method appeared in the literature for the simultaneous determination of the both drugs includes. In literature review we have methods only for the estimation of the above drugs of concern by RP-HPLC individually or in combination with others. In view of the above, a simple and specific analytical method was planned to develop with sensitivity, accuracy, precision and economical.

In the present investigation, RP-HPLC method for the quantitative estimation of Sitagliptin and Simvastatin in bulk drug and pharmaceutical formulations has been developed and validated. The summary results of all validation parameters were expressed in **Table-12**.

The proposed RP-HPLC method is more sensitive, accurate, precise and economic and is suggested for routine analysis of the both drugs in tablet dosage form.

Chapter-7

s*ummary&* conclusion

Summary and conclusion

In the present work, an attempt was made to provide a newer, sensitive, simple, accurate and low cost RP-HPLC method. It is successfully applied for the determination of Sitagliptin and Simvastatin in pharmaceutical preparations without the interferences of other constituent in the formulations.

In this method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to get good optimum results. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, run time etc). The system with Mixed Phosphate Buffer: acetonitrile (25:75) with 0.8 ml/min flow rate is quite robust.

The optimum wavelength for detection was 254nm at which better detector response for the both drugs were obtained. The average retention time for Simvastatin and Sitagliptin were found to be 2.475 and 6.528.

In specificity it is found that there is no interference of any placebo and blank peaks with the drugs of the analysis concern.

The calibration was linear in concentration range of 80-120 µg/ml for Sitagliptin and 16-24 µg/ml for Simvastatin respectively**.** The sensitivity for the both drugs has been calculated and the LOD and LOQ of the Sitagliptin was found to be 3.0 µg and 9.95µg and for Simvastatin was found to be 3.14µg and 10.01µg.

The low values of % R.S.D. indicate the method is precise and accurate. The mean recoveries were found in the range of 99.67 – 100.27 % for Sitagliptin and 99.42 – 100.54% for Simvastatin respectively.Ruggedness of the proposed methods was determined by analysis of aliquots from homogeneous slot by different analysts, using similar operational and environmental conditions; the % R.S.D. reported was found to be less than 2 %.

The proposed method was validated in accordance with ICH parameters and the results of all methods were very close to each other as well as to the label value of commercial pharmaceutical formulation. Therefore, there is no significant difference in the results achieved by the proposed method.

Hence it is suggested that the proposed isocratic RP-HPLC methods can be effectively applied for the routine analysis of Simvastatin and Sitagliptin in tablet formulation.

Chapter-8

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